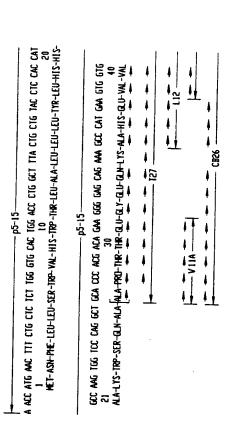
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- (54) Vascular endothelial cell growth factor C subunit.
- (57) Vascular endothelial cell growth factor C subunit DNA is prepared by polymerase chain reaction techniques. The DNA encodes a protein
 that may exist as either a heterodimer or
 homodimer. The protein is a mammalian vascular endothelial cell mitogen and as such is
 useful for the promotion of vascular development and repair. This unique growth factor is
 also useful in the promotion of tissue repair.



EP 0 506 477 A

WL-18-4ET-GJH-ILE-4ET-ANG-ILE-LYS-190-HIS-GJH-SER-GJA-HIS-ILE-GLY-G-U-4ET-SER-+ GIC ACT ATG CAG ATC ATG CGG ATC AAN CCT CAC CAN AGE CAG CAC ATA GGA GAG ATG NAC 120 LYS-CYS SER-CYS-LSY-ASN-11R-ASP-SER ARG-CYS-LSY-ALA-ARG-CAN-LEU-GLU-LEU-ASN-GLU-21 MA TET TOC TICK ANA MAC NOA GAC TOG OGT TICK ANG GOG AGE CAG CTI GAG TIA MAC GAA 8) 6-9-3 - C-E CGT ACT TEC AGA TGT GAC AAG CCA AGG CGG TGA ANG-THR-CTS-ANG-CTS-ASP-LTS-PRO-ANG-ANG-B 200 . - 121 - 67 FIG. 18 FIG.1D TIS THE HELL AND THAT THE COMPANY SETS THE COST TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TCC TGT GTG CCC CTA ATG ANG TIC ATG GAC GTC TAC CAG CGC AGC TAT TOC CGT CCG ATT GAG ACC CTG GTG GAC ATC ISS MIS-CTS-CAU-PID-CTS-SSD-QUI-NGC-NGC-LTS-HIS-LEU-PIG-NG-NG-LTS-HIS-CTS-PID-GH-CTS-TIS-CTS-PID-GH-CTS-TIS-CTS-PID-GH-CT THE CIG CAG CAT AGE MGA TOT GAN TOE AGA CDA ANG ANN GAT MGA MGA MAG CDA GAN ANT 130 PE-LEU GNHIS-SER ARIFETS-GLU-CTS-ABG-PROLIS-LTS-ASP-ARG-TIR-LTS-PRO-GLU-ASP 221 CAC TOT GAG CET TOT TEA GAG CGG AGA AAG CAT TTG TTT GTC CAA GAT CCG CAG AGG TGT 15 01 P. 28 1 21 - BC38 1881 111 - 25-52 -21 . . . - 683

BRIEF DESCRIPTION OF THE DRAWING

Figure 1. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF AA subunit A plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 2. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF AB subunit A plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 3. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF AB subunit B plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 4. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF A 146 amino acid residue subunit SEQ ID NOS:23 & 33.

Figure 5. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF A 190 amino acid residue subunit SEQ ID NOS:30 & 31.

Figure 6. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF A 214 amino acid residue subunit SEQ ID NOS:34 & 35.

Figure 7. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF B 138 amino acid residue subunit SEQ ID NOS:36 & 37.

Figure 8. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF B 158 amino acid residue subunit SEQ ID NOS:38 & 39.

Figure 9. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF C 154 amino acid residue subunit SEQ ID NOS:40 & 41.

BACKGROUND OF THE INVENTION

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A new class of cell-derived dimeric mitogens with apparently restricted specificity for vascular endothelial cells has recently been identified and generally designated vascular endothelial growth factors (VEGFs). The mitogen has been purified from: conditioned growth media of rat glioma cells, [Conn et al., Proc. Natl. Acad. Sci. USA 87: 1323-1327 (1990)]; conditioned growth media of bovine pituitary folliculo stellate cells [Ferrara and Henzel, Biochem. Biophys. Res. Comm. 161: 851-858 (1989) and Gospodarowicz et al., Proc. Natl. Acad. Sci. USA 86: 7311-7315 (1989)]. An endothelial cell growth factor isolated form mouse neuroblastoma cell line NB41 with an unreduced molecular mass of 43-51 kDa and a reduced mass of 23-29 kDa has been described by Levy et al., Growth Factors 2: 9-19 (1989). Connolly et al. (J. Biol. Chem. 264: 20017-20024 [1989]; J. Clin. Invest. 84: 1470-1478 [1989]) describe a human vascular permeability factor that stimulates vascular endothelial cells to divide in vitro and promotes the growth of new blood vessels when administered into healing rabbit bone grafts or rat corneas. An endothelial cell growth factor has been purified from the conditioned medium of the AtT-20 pituitary cell line by Plouet et al., EMBO Jownal 8: 3801-3806 (1989). The growth factor was characterized as a heterodimer composed of subunits with molecular mass of 23 kDa. Leung et al. (Science 246: 1306-1309 [1989]), Keck et al. (Science 246: 1309-1312 [1989]) and Conn et al. (Proc. Natl. Acad. Sci USA 87: 2628-2632 [1990]) have described cDNAs which encode VEGF A which is homologous to the A and B chains of platelet-derived growth factor. Vascular endothelial growth factor I (VEGF I, VEGF AA) is a homodimer with an apparent molecular mass of 46 kDa, with each subunit having an apparent molecular mass of 23 kDa. VEGF I has distinct structural similarities to platelet-derived growth factor (PDGF), a mitogen for connective tissue cells but not vascular endothelial cells from large vessels.

OBJECTS OF THE INVENTION

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It is, accordingly, an object of the present invention to provide novel vascular endothelial growth factor C subunit DNA free of other mammalian DNA. Another object is to provide recombinant genes capable of expressing VEGF C subunit monomer or dimer. Another object is to provide vectors containing the DNA sequences for VEGF A or B plus C subunits. A further object is to provide a host cell transformed with a vector containing the DNA sequence for VEGF A or B plus C or VEGF C alone. It is also an object to provide a recombinant process for making VEGF C subunit. Another object is to provide a novel vascular endothelial cell growth factor which contains the C subunit. This may include heterodimers AC and BC and homodimer CC.

SUMMARY OF THE INVENTION

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Vascular endothelial cell growth factor C subunit DNA is prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and as such is useful for the promotion of vascular development and

repair. This unique growth factor is also useful in the promotion of tissue repair.

DETAILED DESCRIPTION

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The present invention relates to a unique vascular endothelial cell growth factor (designated VEGF), isolated and purified from glioma cell conditioned medium, which exhibits mitogenic stimulation of vascular endothelial cells. Glioma is defined herein as any neoplasm derived from one of the various types of cells that form the interstitial tissue of the central nervous system including brain, spinal cord, posterior bituitary gland and retina. Consequently, the scope of the present invention is intended to include the unique growth factor isolated and purified from any mammalian tissue or other cells including cell lines. Cell lines include, but are not limited to, glioma-derived cell lines such as C6, hs 683 and GS-9L; glioblastomas such as A-172 and T98G; neuroblastomas such as IMR-32 and SK-N-MC; neurogliomas such as H4; tetromas such as XB-2; astrocytomas such as U-87 MG and U-373 MG; embryonal carcinomas and non-transformed glial or astrocyte cell lines, and the human medulloblastoma line TE 671, with GS-9L and TE 671 being preferred. VEGF AB is present and can be isolated from rat tissue including ovary, heart and kidney. Anterior pituitary tumor cell lines such as GH3 and Hs 199 may also be used. It is intended that VEGF of this invention can be obtained from any mammal species capable of producing VEGF, this includes, but is not limited to, rat and numan.

Vascular endothelial cell growth factor may exist in various microheterogeneous forms which are isolated from one or more of the various cells or tissues described above. Microheterogeneous forms as used herein refer to a single gene product, that is a peptide produced from a single gene unit of DNA, which is structurally modified at the mRNA level or following translation. Peptide and protein are used interchangeably herein. The microneterogeneous forms will all have similar mitogenic activities. Biological activity and biologically active are used interchangeably and are herein defined as the ability of VEGF to stimulate DNA synthesis in target cells including vascular endothelial cells as described below which results in cell proliferation. The modifications may take place either in vivo. or during the isolation and purification process. In vivo modification results from, but is not limited to, proteolysis, glycosylation, phosphorylation, deamidation or acetylation at the N-terminus. Proteolysis may include exoproteolysis wherein one or more terminal amino acids are sequentially, enzymatically cleaved to produce microheterogeneous forms which have fewer amino acids than the original gene product. Proteolysis may also include endoproteolytic modification that results from the action of endoproteases which cleave the peptide at specific locations within the amino acid sequence. Similar modifications can occur during the purification process which also results in production of microheterogeneous forms. The most common modification occurring during purification is proteolysis which is generally held to a minimum by the use of protease inhibitors. Under most conditions one or more microheterogeneous forms are present following purification of native VEGFs. Native VEGFs refers to VEGF isolated and purified from cells that produce VEGFs. Vascular endothelial cell growth factor may also exist in various alternatively spliced forms which is defined herein as the production of related mRNAs by differential processing of exons and introns. Exons are defined as those parts of the DNA sequence of a eukaryotic gene that code for the final protein product. It is also intended that the present invention includes VEGF subunits A.B and C which are defined as comprising the full length translation products of all alternatively spliced mRNAs made from the gene encoding the subunits and their corresponding mature amino acid sequences generated by proteolytic removal of the amino terminal secretory leader amino acid sequences. It is further intended that the invention only include those microheterogeneous and alternatively spliced VEGF subunits which when in the dimeric form exhibit biological activity such as vascular endothelial cell stimulation as discussed below.

Glioma cells such as the rat cell line GS-9L are grown to confluence in tissue culture flasks, about 175 cm², in a cell culture medium such as Dulbecco's Modified Eagle's Medium (DMEM) supplemented with about 10% newborn calf serum (NCS). When the cells reach confluence the culture medium is removed, the cell layers are washed with Ca⁻⁻, Mg⁻⁻-free phosphate buffered saline (PBS) and are removed from the flasks by treatment with a solution of trypsin, about 0.1%, and EDTA, about 0.04%. The cells, about 1 x 10³, are pelleted by centrifugation, resuspended in about 1500 ml of DMEM containing about 5% NCS and plated into a ten level cell factory (NUNC), 6,000 cm² surface area. The cells are incubated for about 48 to about 96 hours, with 72 hours preferred, at about 37° C in an atmosphere of about 5% CO₂. Following incubation the medium is removed and the cell factories are washed about 3 times with PBS. About 1500 ml of fresh culture media is added containing about a 1:2 mixture of Ham's-F12/DMEM containing about 15 mM Hepes, pH about 7.4, about 5 μg/ml insulin, about 10 μg/ml transferrin and with or without about 1.0 mg/ml bovine serum albumin. This medium is replaced with fresh medium after about 24 hr and collected every 48 hr thereafter. The collected conditioned medium is filtered through Whatmen #1 paper to remove cell debris and stored at about -20° C.

The GS-9L conditioned medium is thawed and brought to pH 6.0 with 1 M HCl. The initial purification step consists of cation exchange chromatography using a variety of cation exchangers on a variety of matrices such

as CM Sephadex C-50, Pharmacia Mono S. Zetachrom SP and Polyaspartic Acid WCX (Nest Group) with CM Sephadex C-50 (Pharmacia) being preferred. The VEGF-containing culture medium is mixed with CM Sephadex C-50 at about 2 gm per about 20 L of the conditioned medium and stirred at low speed for about 24 hr at 4° C. The resin is allowed to settle and the excess liquid is removed. The resin slurry is packed into a column and the remaining culture medium is removed. Unbound protein is washed from the column with 0.05 M sodium phosphate, about pH 6.0, containing 0.15 M NaCl. The VEGF AB is eluted with about 0.05 M sodium phosphate, about pH 6.0, containing about 0.6 M NaCl.

The active fractions collected from the CM Sephadex C-50 column are further fractionated by lectin affinity chromatography for additional purification of VEGF AB. The lectins which may bind VEGF AB include, but are not limited to, lectins which specifically bind mannose residues such as concanavalin A and lens culinaris agglutinin, lectins which bind N-acetylglucosamine such as wheat germ agglutinin, lectins that bind galactose or galactosamine and lectins which bind sialic acids, with concanavalin A (Con A) being preferred. A 0.9 cm diameter column containing about 5 ml packed volume of Con A agarose (Vector Laboratories) is washed and equilibrated with about 0.05 M sodium acetate, about pH 6.0, containing about 1 mM CaCl₂, about 1 mM MnCl₂ and about 0.6 M NaCl. The unbound protein is washed from the column with equilibration buffer. The VEGF AB is eluted with about 0.1 M NaCl buffer containing about 0.32 M α-methyl mannoside and about 0.28 M α-methyl glucoside.

The VEGF AB active eluate from the Con-A column is applied to a Polyaspartic Acid WCX cation exchange high performance liquid chromatography (HPLC) column, 4.6 mm x 250 mm, pre-equilibrated in about 0.05 M sodium phosphate buffer, pH 6.0. The column is eluted with a linear gradient of about 0 to 0.75 M NaCl in the phosphate buffer over about 60 minutes. The flow rate is maintained at about 0.75 ml/min collecting 0.75 ml fractions. Vascular endothelial cell growth factor AB activity is present in fractions eluting between approximately 21.7 and 28.5 ml.

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The active fractions eluted from the polyaspartic WCX column that contain VEGF AB are pooled, adjusted to about pH 7.0 and loaded onto a 1 x 10 cm column of Pharmacia Chelating Sepharose 6B charged with an excess of copper chloride and equilibrated in about 0.05 M sodium phosphate, about pH 7.0, containing about 2 M NaCl and about 0.5 mM imidazole (A buffer). VEGF AB is eluted from the column with a gradient from 0-20% B over 10 minutes, 20-35% B over 45 minutes and 35-100% B over 5 minutes at a flow rate of 0.3 ml/min, where B buffer is 0.05 M sodium phosphate, pH 7.0, containing about 2 M NaCl and 100 mM imidazole. The active fractions containing VEGF AB activity eluted between about 12.6 and 22.8 ml of the gradient effluent volume.

The pooled fractions containing VEGF AB activity eluted from the metal chelate column are loaded onto a 4.6 mm x 5 cm Vydac

 C_4 reverse phase HPLC column (5 μ m particle size) previously equilibrated in solvent A [0.1% trifluoroacetic acid (TFA)]. The column is eluted with a linear gradient of about 0 to 30% solvent B over 15 minutes, 30% B for an additional 15 minutes, then 30-45% B over 22.5 minutes and finally 45-100% B over 5.5 minutes. Solvent B consists of solvent A containing 67% acetonitrile (v/v). The bow rate is maintained at about 0.75 ml/min and fractions are collected every minute. The homogeneous VEGF AB elutes from the C_4 column under these conditions at between about 32 and about 38 ml of the gradient effluent volume.

Purity of the protein is determined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in 12.5% crosslinked gels using the technique of Laemmli, Nature 227:680-684 (1970). The silver stained gels show VEGF AB to consist of one band under non-reducing conditions with an approximate apparent molecular mass of about 58,000 daltons. When a sample containing the microheterogeneous forms of VEGF AB is separated under reducing conditions it migrates as two about 23 kilodalton (kDa) subunits. The purification process results in VEGF AB that is essentially free of other mammalian cell products, such as proteins. Recombinantly derived VEGF AB will also be free of mammalian cell products.

Biological activity is determined by mitogenic assay using mammalian vascular endothelial cells. Human umbilical vein endothelial (HUVE) cells are plated on gelatin-coated dishes at a density of about 5000 cells per well in about 500 μl of Medium 199 (M199) containing about 20% heat-inactivated fetal calf serum (FCS). Samples to be assayed are added at the time of plating. The tissue culture plates are incubated at about 37° C for about 12 hours and about 2 microcuries of tritiated thymidine (NEN, 20 Ci/mmol) is added per ml of assay medium (1.0 μCi/well). The plates are incubated for a further 60 hr, the assay medium is removed and the plates are washed with Hanks balanced salt solution containing about 20 mM Hepes, about pH 7.5, and about 0.5 mg/ml bovine serum albumin. The cells are lysed and the labelled DNA solubilized with about 200 μl of a solution containing about 2 gm of sodium carbonate and about 400 mg sodium hydroxide in about 100 ml water. The incorporated radioactivity was determined by liquid scintillation counting. The concentration of VEGF which elicited a half-maximal mitogenic response in HUVE cells was approximately 2 ± 1 ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 μg/ml to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by VEGF AB.

A purified about 1-2 μg sample of VEGF AB is reduced in about 0.1 M Tris, about pH 9.5, with about 0.1% EDTA, about 6 M guanidinium chloride and about 20 mM dithiothreitol for about 2 hr at about 50°C. The reduced protein is carboxymethylated for about 1 hour in a solution containing about 9.2 μM of unlabelled and 2.8 μM of ¹⁴C-iodoacetic acid in about 0.7 M Tris, about pH 7.8, and about 0.1% EDTA and about 6 M guanidinium chloride. The protein is carboxymethylated for about 1 hr at room temperature. The protein is isolated after reduction and carboxymethylation by reverse phase HPLC chromatography on a Vydac C₄ column, about 4.6 mm x 5 cm. The protein subunits are loaded onto a column pre-equilibrated with about 0.1% TFA and eluted by a 45 ml linear gradient from about 0.1% TFA to 0.1% TFA/67% acetonitrile at a bow rate of about 0.75 ml/min. The reduced and carboxymethylated protein eluted as two peaks at approximately 23 and 25 ml with the proportion being approximately equal as determined by monitoring absorbance at 210 nm.

Samples of the reduced and carboxymethylated monomers are applied to polybrene-coated glass fiber filters and their N-terminal sequences are determined by Edman degradation in an ABI gas phase microsequencer in conjunction with an ABI 120A on line phenylthiohydantoin analyzer following the manufacturers instructions. The protein showing the peak of absorbance eluting at approximately 25 ml (A subunit or monomer) yielded an amino terminal sequence of: SEQ ID NO:1

Ala Pro Thr Thr Glu Gly Glu Gln Lys Ala His Glu Val Val which is identical to the A chain monomers of VEGF AA, Conn et al., Proc. Natl. Acad. Sci. USA 87: 2628-2632 (1990). The peak of absorbance eluting at approximately 23 ml (B subunit or monomer) yielded an N-terminal sequence of: SEQ ID NO:2

Ala Leu Ser Ala Gly Asn Xaa Ser Thr Ser Thr Glu Met Glu Val Val

Pro Phe Asn Glu Val

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plus a nearly equal amount of a truncated form of the same sequence missing the first three amino acid residues. The missing Xxx residue corresponds to an Asn residue in the cloned cDNA, see below. Since this missing Asn occurs in a classical Asn Xxx Ser/Thr N-glycosylation sequence it is presumed to be glycosylated. The A subunit and the total of both B subunits are recovered in nearly equal amounts supporting the interpretation that the two peptides combine to form an AB heterodimer in VEGF AB.

A sample of the A monomer was treated with either the protease trypsin which cleaves polypeptides on the C-terminal side of lysine and arginine residues or Lys C which cleaves polypeptides on the C-terminal side of lysine by procedures well known in the art. The peptides are isolated by reversed phase - HPLC(RP-HPLC). The amino acid sequences of the isolated peptides are determined using the Edman degradation in the ABI gas phase sequenator in conjunction with the ABI 120 A on line phenylthiohydantoin analyzer following manufacturer's instructions. The amino acid sequences are shown in Figure 1.

Reduced and carboxymethylated A monomer is dried and solubilized in about 0.7 M Tris, about pH 7.8, about 6 M guanidinium chloride containing about 0.1% EDTA. V8 protease is added in 0.1 M ammonium bicarbonate buffer, about pH 8.0, and the mixture is incubated for about 48 hr at about 37°C. The protease cleaves predominantly on the carboxyl terminal side of glutamic acid residues. The resulting polypeptides were resolved by C₁₈ RP-HPLC as above.

The reduced and carboxymethylated A subunit protein solution is adjusted to a pH of about 6.8 with 6 N HCI and dithiotreitol is added to a final concentration of 2 M for reduction of any methionine sulfoxide to methionine residues. After about 20 hr of reduction at about 39°C the protein is repurified by C₄ HPLC. The product is dried and cleaved on the carboxyl terminal side of methionine residues by 200 µl of 40 mM cyanogen bromide in about 70 % (v/v) formic acid under an argon atmosphere at about 20°C for about 24 hr in the dark. The cleavage products are resolved by C₁₈ RP-HPLC. The amino acid sequence is shown in Figure 1, see Conn et al., Proc. Natl. Acad. Sci USA 87:2628-2632 (1990).

The full length 190 amino acid residue protein translation product of the VEGF AB, A monomer or subunit, which is now known to be identical with the VEGF AA, A monomer, and its cDNA coding sequence are shown in Figures 2 and 6. The mature amino terminus begins at residue 27, immediately following a typical hydrophobic secretory leader sequence. A single potential N-glycosylation site exists at Asn₁₀₀. Most (143 amino acid residues) of the 164 residues of the reduced and carboxymethylated mature subunit including the amino terminus and HPLC reversed phase-purified products of tryptic (T), Lys-C (L), Staphylococcus aureus V8 protease (V8) and cyanogen bromide (CB) cleavages, were determined by direct microsequencing (Applied Biosystems 470A) using a total of 5 μg of protein. All residues identified by amino acid sequencing are denoted by arrows pointing to the right either directly beneath the mature processed sequence following the bracket at residue 27 for the amino terminal determination of the whole subunit or, for residues identified from the polypeptide cleavage products, above the double-headed arrows spanning the length of the particular polypeptide. One listed pair of polypeptides, V18A and V18B, was sequenced as a mixture and, therefore, are only confirmatory of the cDNA-deduced amino acid sequence, see Figures 1 and 5.

Samples of the reduced and carboxymethylated pure VEGF AB, A and B monomers, were each digested

with the Lys-C endoproteinase, which cleaves polypeptides on the C-terminal side of lysine residues. The pertides were isolated by reverse phase HPLC and their amino acid sequences were determined as described above. The locations of the peptides in the final VEGF AB. A and B sequences are shown in Figure 2 and Figure 3, respectively.

The full length coding region of the A subunit or monomer is determined from three sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences Phe-Met-Asp-Vai-Tyr-Gin from polypeptide L42 (residues 42-47) and Cys-Lys-Asn-Thr-Asp from polypeptide 138 (residues 42-47) and Cys-Lys-Asn-Thr-Asp from polypeptide 138 (residues 42-47) and central region of the cDNA for VEGF A chain following the procedure of Saiki et al., Science 230: 1350- 1354 (1985). A single band migrating at 420 bp was gel purflied, discedure of Saiki et al., Science 230: 1350- 1354 (1985). A single band migrating at 420 bp was gel purflied, discedure of Saiki et al., Science 230: 1350- 1354 (1985). A single band migrating at 420 bp was gel purflied, disceded with Sail, ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (p4238) was used described by Frohman et al. Proc. Natl. Acad. Sci. USA 85:8998-9002 (1988). These 5' and 3' clones are defermined by Frohman et al. Proc. Natl. Acad. Sci. USA 85:8998-9002 (1988). These 5' and 3' clones are defermined and pW3, respectively. Regions of complete DNA sequences, excluding the primers, determined for each set of clones are indicated by double-headed arrows above the nucleotide sequence. In addition to conding the 164 amino acid secreted form identified by protein sequencing, two alternatively spliced cDNAs encoding at 146 amino acid secreted form identified by protein sequencing, two alternatively spliced cDNAs encoding at 146 amino acid secreted form identified by protein sequencing, two alternatively spliced conding and a 214 amino acid secreted form identified by protein sequencing, two alternatively spliced conding at 146 amino acid secreted form identified by protein sequencing, two alternatively spliced conding at 146 amino acid secreted form identified by protein sequencing, two alternatively and the conding at 146 amino acid secreted form identified by protein sequenced. Figures 4, 5 and 5 an

The full length coding region of the B subunit or monomer is determined from four sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences from polypeptide L50 are used to PCR amplify the central region of the cDNA for VEGF AB, B monomer, following the procedure of Saiki et al., Science 230: 1350-1354 (1985). A single band migrating at 108 bp was gel purified, digested with Sall. ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (pYG) was used to design antigeated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (pYG) was used to design antigence and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol described by Frohman et al. Proc. Natl. Acad. Sci. USA 85:8998-9002 (1988). These 5' and 3' clones are denoted p5V2 and p3V2, respectively. Additional 5' end sequences are determined from clone 202 isolated from a cDNA library prepared from GS-9L poly Ar RNA. Regions of complete DNA sequences. excluding the primers. determined for each set of clones are indicated by double-headed arrows above the nucleotide sequence. The entire mined for each set of clones are indicated by double-headed arrows above the nucleotide sequence. The entire passe sequence for the 158 amino acid microheterogeneous B subunit are shown in Figures 7 and 8.

The full length coding region of the C subunit or monomer is determined from three sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequence Phe Ser Pro Ser Cys Val and Glu Met Thr Phe Ser Gly from rat VEGF B subunit are used to PCR amplify the central region of the cDNA of VEGF C chain following the procedure of Saiki et al., Science 230: 1350-1354 (1985). A band migrating at 180 bp is gel purified, reamplified and digested with Sail, ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (pFSEM') is used to design antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol described by Frohman et al., Proc. Natl. Acad. Sci. USA 85: 8998-3' ends of the cDNA according to the protocol described by Frohman et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988). The 5' and 3' clones are denoted p5:16 and p3:19, respectively. The entire base sequence and amino acid sequence for the C subunit are shown in Figure 9.

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It is intended that vascular endothelial cell growth factor of the present invention exist as a heterodimer consisting of an A microheterogeneous and/or alternatively spliced subunit or a B microheterogeneous and/or alternatively spliced subunit combined with a C microheterogeneous and/or alternatively spliced subunits. The native forms of the A. B. C subunits may be processed form alternatively spliced full length translation products. The heterodimers or heterodimeric species can be depicted as: A+B, A+C or B+C with the A. B or C subunits existing in any of the alternatively spliced or microheterogeneous forms. The homodimers or homodimeric species can be formed by combinations of any of the alternatively spliced or microheterogeneous forms. The homodimers or homodimeric species can be formed by combinations of any of the alternatively spliced or microheterogeneous forms. It is also intended that the invention include all of the individual subunit forms of the A subunit, the B subunit and the C subunit of VEGF.

It is further intended that the nucleotide sequence for vascular endothelial cell growth factor be interpreted to include all codons that code for the appropriate amino acids in the sequence for each of the vascular endothelial growth factor subunits, as indicated by the degeneracy of the genetic code. It is further intended that the nucleotide sequence and the amino acid sequence for VEGF subunits include truncated genes or proteins that result in proteins which exhibits biological activity similar to vascular endothelial cell growth factor. The scope of the invention is intended to include all naturally occurring mutations and allelic varients and any tandomly generated artifical mutants which may change the sequences but do not alter biological activity as dedomly generated artifical mutants which division of vascular endothelial cells.

The above described heterodimers, homodimers and subunits of vascular endothelial cell growth factor are characterized by being the products of chemical synthetic procedures or of procaryotic or eucaryotic host

ressed in E. coli. Other expression systems and host cells are well known in the art. commercially available plasmid such as pKK 223-3 (Pharmacia) as modified as by Linemeyer \underline{et} \underline{al} , and expby Linemeyer et al., European Patent Application, Publication No. 259,953. The cDNA is incorporated into a cDNA for the monomemor forms of the A. B and C subunits can be expressed in a system such as that described and vector systems along with methods for the introduction of recombinant vectors into mammalian cells. The Associates and Wiley-Interscience, 1987 and supplements, disclose various mammalian expression vectors Laboratory Press (1989) and Cwrent Protocols In Molecular Biology, Ausubel et. al. Eds. Greene Publishing art. Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Edition, Book 3, Cold Springs Harbor or viruses and cosmids. The expression of mammalian genes in cultured mammalian cells is well known in the may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors ANS sequence that directs RNA polymerase to bind to DNA and to initiate RNA promoter is defined as a DNA sequence that directs useful restriction enzyme sites, a high copy number, strong promoters and efficient translational stop signals. tain: an origin of replication for autonomous replication in host cells, selective markers, a limited number of teria-yeast, bacteria-plant or bacteria-animal cells. An appropriately constructed expression vector anound cona number of virus expression systems. Specifically designated vectors allow the shuttling of DNA between baccells and animal cells, with mammalian cells being preferred. The genes may also be expressed using any of be used to express genes in a variety of hosts such as bacteria, biuegreen algae, yeast cells, insect cells, piant binant DNA sequences or genes and the translation of their mRNAs in an appropriate host. Such vectors can vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of recomby a number of different host cells which contain at least one of a number of expression vectors. Expression rated in an oligomeric unit. Expression of the recombinant VEGF genes (recombinant DNA) is accomplished expression of the DNA sequences as described herein. A monomer is defined as a subunit that is not incorpo-

The high Cys content and glycoslystion sites of the A, B and C subunits along with the structure of the homo-ression may be carried out in Chinese hamster ovary (CHO) cells with the cloned VEGF DNA cotransfected with the gene encoding dihydrofolate reductase (dhfr) into dhfr- CHO cells, see Sambrook et al. Transformants expressing dhfr are selected on media lacking nucleosides and are exposed to increasing concentrations of methorexate. The dhfr and VEGF genes are thus coamplified leading to a stable cell line capable of expressing methorexate. The dhfr and VEGF genes are thus coamplified leading to a stable cell line capable of expressing thigh levels of VEGF. The plasmid is designed to encode either an A subunit, a B subunit or a C subunit or a Combination of any two of these subunits. The two cDNAs are operably attached so that the protein produced will be dimeric and will have VEGF biological activity. Operably attached refers to an appropriate sequential will be dimeric and will have vector containing the operably attached genes, cDNA segments or nucbroadement of nucleotide segments. CDNA segments or genes auch that the desired protein will be produced by cells containing an expression vector containing the operably attached genes, cDNA segments or nucleotides. Plasmids containing a single subunit species may be used to cotransfect a suitable cell line.

The expressed proteins (homodimers or heterodimers) are isolated and pwified by standard protein purification processes. It is to be understood that the expression vectors capable of expressing heterodiment forms of VEGF will contain two DNA sequences which will encode either an A subunit and/or a DNA sequence which will encode a C subunit. Expression vectors capable of expressing homodimeric forms of VEGF will contain either one or two DNA sequences which encode either

two A, two B or two C subunits.

The ability of the various species of VEGF to stimulate the division of vascular endothelial cells makes this protein in all microheterogeneous forms and alternative splicing forms as previously described. The protein as used herein is intended to include all microheterogeneous forms as previously described. The protein can be used to treat wounds of mammals including humans by the administration of the novel protein to patients

in need of such bestment.

The novel method for the stimulation of vascular endothelial cells comprises beating a sample of the desired vascular endothelial cells in a nutrient medium with mammalian VEGF, prefetably human or rat, at a concentration of about 1-10 ng/ml. If the vascular endothelial cell growth is conducted in vitro. The process requires the presence of a nutrient mediuin such as DMEM or a modification thereof and a low concentration of calf or the presence of a nutrient mediuin such as Dovine serum auch as about 0 to 2% by volume. Preservatives such as antibiotics may also be included; these bovine serum such as about 0 to 2% by volume. Preservatives such as antibiotics may also be included; these

are well known in the art.

The novel growth factors of this invention are useful for the coverage of artificial blood vessels with vascular endothelial cells. Vascular endothelial cells from the patient would be obtained by removal of a small sequent of peripheral blood vessel or capillary-containing tissue and the desired cells would be grown in culture in the presence of VEGF and any other supplemental components that might be required for growth. After growth of adequate numbers of endothelial cells in culture to cover a synthetic polymeric blood vessel the cells would adequate numbers of endothelial cells in culture to cover a synthetic polymeric blood vessel the cells would be plated on the inside surface of the vessel, such as fixed umbilical vein, which is then implanted in the patient.

to be neovascularized either from implanted slow release polymenic material or from slow release pumps or ug/kg/day of body weight. For internal vascular growth, the formulation would be released directly into the region to about 1 mg/cm²/day. For vascular repair VEGF is given intraveneously at a rate of about 1 mg to about 100 cularization and healing of surface wounds the formulation would be applied directly at a rate of about 10 ng used either topically for tissue repair or intravascularly for vascular repair. For applications involving neovasused to induce and promote growth of tissue by inducing vascular growth and for repair. The peptide can be The novel peptides can also be used for the induction of tissue repair or growth. The pure VEGF would be surface and multiple layers of smooth muscle cells cover the outside, the vessel is implanted into the patient. endothelial cells on the interior surface. Once the endothelial cells form a confluent monolayer on the inside thread) would be used to support the culture growth of the smooth muscle cells on the exterior side and vascular with or without a coating of proteins, or a non-immunogenic biopolymeric material such as surgical suture procedures well known in the art. A tubular mesh matrix of a biocompatible polymer (either a synthetic polymer, would be grown in the presence of VEGF as outlined above. The smooth muscle would be grown in culture by smooth muscle cells from the patient would be obtained and grown separately in culture. The endothelial cells The novel proteins are also used for the production of artificial vessels. Vascular endothelial cells and surface of the artificial vessel and thereby decrease the tendency of vessel blockage or embolism elsewhere. patible. The non-thrombogenic endothelial cell lining should decrease the incidence of clot formation on the surgically implanted into the patient and, being lined with the patients own cells, would be immunologically comformed to enhance attachment of the cells to the artificial surface. The cell-lined artificial vessel would then be either covalently or noncovalently, with proteins such as fibrin, collagen, fibronectin or laminin would be perplantation endothelial cells migrate into and grow on the artificial surface. Prior coating of the artificial vessel Alternatively, tubular supports are coated in vitro with VEGF prior to implantation into a patient. Following im-

For non-topical application the VEGF is administrated in combination with pharamaceutically acceptable carri era or diluents such as, phosphate buffer, saline, phosphate buffered saline, Ringer's solution, and the like, in a pharamaceutical composition, according to standard pharamaceutical practice. For topical application, various pharmaceutical formulations are useful for the administration of the active compound of this invention. Such formulations include, but are not limited to, the following: ointments such as hydrophilic petrolatum or polyethylene glycol ointment; pastes which may contain poms such as xanthan gum; solutions such as alcoholic polyethylene glycol ointment; gels such as aluminum hydroxide or sodium alginate gels; albumins such as alcoholic or aqueous solutions; gels such as aluminum hydroxide or sodium alginate gels; albumins such as alcoholic or agueous solutions; gels such as aluminum hydroxide or sodium alginate gels; albumins such as alcoholic alluloses and alkylhydroxyalkyl celluloses, for example methylcellulose, hydroxyethyl celluloses, for example methylcellulose, hydroxyethyl celluloses, for example methylcellulose, hydroxyethyl celluloses, for example methylcellulose; polyoxamers such as Pluboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl celluloses, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose, polyoxamers such as Pluboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose, polyoxamers such as solutions. Pluboxymethyl cellulose, polyoxamers such as solutions and algorithms and allowed and allowe

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

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40 Preparation of Medium Conditioned By GS-9L Cells

GS-9L cells were grown to confluence in 175 cm² tissue culture flasks in Dulbecco's Modified Eagle's Medium\10% newborn calf serum (DMEM\NCS). At confluence the medium was decanted from the flasks, the flasks were washed with calcium and magnesium free phosphate buffered saline (PBS) and the cells were re-moved by treatment with a 1X solution of trypsin\EDTA (Gibco). The cells (1 x 10⁸) were pelleted by centrifugation, resuspended in 1500 ml of DMEM\5% NCS and plated into a ten level (6000 cm² surface area) cell factory (NUNC). After 72 hours incubation at 37° C in a 5% CO₂ atmosphere the medium was decanted and the cell factories were washed 3 times with PBS. The cells were refed with 1500 ml of a 1:2 mixture of Ham's the cell factories were washed 3 times with fresh F-12\DMEM after 24 hours and 1.0 mg/ml bovine serum albumin. This medium was changed with fresh F-12\DMEM after 24 hours and collected every 48 hours after that. The conditioned medium was filtered through a Whatman #1 paper to remove cell debris and stored frozen at -20°C.

EXAMPLE 2

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Carboxymethyl-Sephadex Chromatography of VEGF AA and VEGF AB

of CM Sephadex C-50 cation exchange (Pharmacia) resin preequilibrated in PBS adjusted to pH 6.0 with 1 M HCI were added to 20 liters of conditioned medium. The mixture was stirred at low speed for 24 hours at 4° C. The resin was then allowed to settle and the medium was siphoned off. The remaining resin alow protein was into a 3.0 cm diameter column and any remaining medium was allowed to drain off. Unbound protein was washed off the column with 0.05 M sodium phosphate, pH 6.0, containing 0.15 M NaCl. Vascular endothelial growth factor activity was eluted from the column with a subsequent wash of 0.05 M sodium phosphate, pH 6.0, containing 0.6 M NaCl.

EXAMPLE 3

A 0.9 cm dismeter column containing about 5 ml of packed Con A agarose (Vector Laboratories) was equilibrated with 0.05 M sodium acetate, pH 6.0, containing 1 mM Ca**, 1 mM Mn** and 0.6 M MaCl. The active eluate from the CM Sephadex C-50 column, Example 2, was applied to the Con A agarose and unbound protein was from the CM Sephadex C-50 column. Example 2, was applied to the Con A agarose and unbound protein was washed from the column volumes of washed from the column with equilibration buffer. The column was then rinsed with three column volumes of washed from the column with equilibration buffer. The column was then three column with equilibration buffer.

washed from the column with equinotation buriet. The column was then this decide with MaCl. Bound protein was subsocioum acetate, pH 6.0, containing 1 mM Ca⁺, 1 mM Mn⁺ and 0.1 M MaCl. Bound protein was subsequently eluted from the column by application of this buffer supplemented with 0.32 M α-methyl mannoside and 0.28 M α-methyl glucoside.

EXAMPLE 4

Polyaspartic Acid WCX HPLC Cation Exchange Chromatography of VEGF AA and VEGF AB

Concanavalin A (Con A) Lectin Affinity Chromatography of VEGF AA and VEGF AB

The active eluste from the Con A column, Example 3, was applied to a 25 cm x 4.6 mm poly(aspartic acid) WCX cation exchange HPLC column (Nest Group) pre-equilibrated in 0.05 M sodium phosphate buffer, pH 6.0. The column was eluted with a linear gradient of 0 to 0.75 M NaCl in this buffer over 60 minutes at a flow rate The column was eluted with a linear gradient of 0 to 0.75 M NaCl in this buffer over 60 minutes at a flow rate of 0.75 ml/min collecting 0.75 ml fractions. VEGF AB activity present in fractions eluting between approximately of 0.75 ml/min collecting 0.75 ml fractions.

EXAMPLE 5

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οz

Metal Chelate Chromatography

21.7 and 28.5 ml were pooled.

The scrive fractions eluted from the poly(aspartic scid) WCX column, Example 4, that contain VEGF AB were pooled, adjusted to pH 7.0 and loaded onto a 1 x 10 cm column of Pharmacia Chelating Sepharose 6B charged with an excess of copper chloride and equilibrated in 0.05 M sodium phosphate, pH 7.0, containing 2 M NaCl and 0.5 mM imidazole (A buffer). VEGF AB was eluted from the column with a gradient from 0-20% B over 10 minutes at a flow rate of 0.3 ml/min, where B buffer was 0.05 M sodium phosphate, pH 7.0, containing 2 M NaCl and 100 mM imidazole. The active fractions containing VEGF AB activity eluting between 12.6 and 22.8 ml of the gradient effluent volume were pooled.

45 Reverse Phase Chromatography

EXAMPLE 6

The fractions containing VEGF AB activity pooled from the metal chelate column, Example 5 were loaded onto a 4.6 mm x 5 cm Vydac C₄ reverse phase HPLC column (5 µm particle size) equilibrated in solvent A (0.1% brith united in solvent B over 15 minutes, 30% B big tot an additional 15 minutes, then 30-45% B over 22.5 minutes and finally 45-100% B over 5.5 minutes where solvent B = A containing 67% acetonitrile. The flow rate was maintained at 0.75 ml/min. The active VEGF AB solvent B = A containing between approximately 32.2 and 37.5 ml of the gradient effluent volume were pooled.

EXAMPLE 7

Mitogenic Assays

Human umbilical vein endothelial cells (HUVE) were plated on gelatin-coated 48 well tissue culture dishes

at a density of 5000 cells/well in 500 µl of Medium 199 containing 20% heat inactivated fetal calf serum (FCS). Samples to be assayed were added at the time of plating. The tissue culture plates are incubated at 37° C for 12 hours and 2 microcuries of britisted thymidine (NEM, 20 Ci/mmol) was added per ml of assay medium (1.0 µCi/vell). The plates were incubated for a further 60 hr, the assay medium was removed and the plates were ucubated for a further 60 hr, the assay medium was removed and the plates were with Hanks balanced salt solution containing 20 mM Hepes, pH 7.5, and 0.5 mg/ml bovine serum albumin. The cells were lysed and the labelled DNA solubilized with 200 µl of a solution containing 2 gm of sodium min. The cells were lysed and the labelled DNA solubilized with 200 µl of a solution containing 2 gm of sodium incurting 2 gm of sodium cantoning.

The concentration of VEGF AB which elicited a half-maximal mitogenic response in HUVE cells was approximately $\Sigma \pm 1$ ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 $\mu g/ml$ to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by VEGF AB.

EXAMPLE 8

SI

Purity And Protein Structural Characterization of VEGF AB

Purity of the protein under non-reducing conditions was determined by SDS-PAGE in 12.5% crosslinked gels according to the method of Laemmli, Nature 227: 680-685 (1970). The silver-stained gel contained a single band with an apparent mass of approximately 58 kDa. VEGF AB migrated in SDS-PAGE under reducing conditions in 15% crosslinked gels as a broad silver-stained band with apparent molecular mass of approximately 23 kDa.

VEGF AB was stored a 4°C in the aqueous trifluoroscetic scid (TFA)/scetonitrile mixture used to elute the homogeneous protein in reversed phase C₄ HPLC chromatography at the final stage of the purification protocol previously described. Aliquots of the purified protein (1-2 μg) were vacuum evaporated to dryness in acid-washed 10 x 75 mm glass tubes and reduced for 2 hours at 50°C in 100 μl of 0. 1 M Tris buffer, pH 9.5, and 6 M guanidinium chloride containing 0.1% EDTA and 20 mM dithiothreitol (Calbiocchem, Ultrol grade) under an argon atmosphere. The reduced protein was subsequently carboxymethylated for 1 hour at 20°C by the addition of 100 μl of 0.7 M Tris, pH 7.8, containing 0.1% EDTA, 6 M guanidinium chloride, 9.2 μM unlabeled iodoscetic acid and 50 μCi of iodo[2-1* C]scetic acid (17.9 mCi/mmole, Amersham). After completion of the carboxymethylated protein was repurified by elution of the carboxymethylated protein was repurified by elution with a 45 minute linear gradient, the mixture was loaded directly onto a 4.6 mm x 5.0 cm Vydac C₄ column which had been prequilibrated in 0.1% TFA at a flow rate of 0.75 ml/min and stored in this elution solution dient of 0 to 67% (viv) acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min and stored in this elution solution at 4°C. The reduced and carboxymethylated protein eluted as two peaks at approximately S3 and S5 ml that at 4°C. The reduced and carboxymethylated protein eluted as two peaks at approximately squal area as determined by monitoring absorbance at 210 nm.

Samples of the two protein subunits isolated after reduction and carboxymethylation were each applied to polybrene-coated glass fiber filters and their N-terminal sequences were determined by Edman degradation in an NBI gas phase microsequencer in conjunction with an ABI 120A on line phenylthiohydantoin analyzer following manufacturers instructions. The peak of absorbance eluting at approximately 23 ml (B subunit) yielded an amino terminal sequence Ala Pro Thr Thr Glu Gly Glu Gln Lys Ala His Glu Val Val SEQ ID NO: 1 identical to VEGF AA. The peak of absorbance eluting at approximately 23 ml (B subunit) yielded the N-terminal sequence Ala Cla Ser Ala Gly Asn Xas Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val SEQ ID NO: 2 plus a neastly equal amount of a truncated form of the same sequence missing the first three residues. The missing X residue corresponds to an Asn in the cloned sequence. Since this missing Asn occurs in a classical Asn-X-Ser/Thr N-glycosylation sequence it is presumed to be glycosylated. The A and sum of the B chain peptides servered in nearly equal amounts supporting the interpretation that the two peptides combine to form AB heterodimer in VEGF II.

Reduced and carboxymethylated A and B subunits (650 ng each) were each dried by vacuum evaporation in acid-washed 10 x 75 mm glass tubes. Lys C protease (50 ng, Boehringer Mannheim), an enzyme that cleaves on the carboxyl terminal side of lysine residues, was added to each tube in 100 µl of 25 mM Tris, pH 8.5, 0.1 % EDTA. The substrate protein subunits were separately digested at 37°C for 8 hours and the resulting polypeptides resolved by reversed phase HPLC chromatography on a 4.6 mm x 25 cm Vydac C₁₈ column equilibrated in 0.1% TFA. Polypeptides were fractionated by elution with a 2 hour linear gradient of 0-67% acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min at 20°C. Individual peaks were manually collected and stored in this elution solution at 4°C.

The amino acid sequences of the isolated peptides were then determined using Edman degradation in an BI gas phase sequenator in conjunction with the ABI 120 A on line phenylthiohydantoin analyzer (Applied Biosystems Int.). The peptide sequences are shown in the following Figures 2 and 3. The amino acid sequence

of VEGF AB mature B subunit in the heterodimer is the 135 amino acid form derived from the 158 full length the 164 amino acid form. The amino acid sequence of Lys C fragment L26 (Fig. 3) demonstrates that the form of Lys C fragment L20 (Fig. 5) demonstrates that the form of VEGF AB mature A subunit in the heterodimer is

amino acid form.

EXAMPLE 9

Cloning and Sequencing of the VEGF A Monomer

PCR Amplification, Cloning and Sequencing of P4238

quences of VEGF A subunit between LysC fragment L 42 and tryptic fragment T38. These oligonucleotides

L42.2 5' TTTGTCGACTT[TC]ATGGA[TC]GT[N]TA[TC]CA 3'

Two degenerate oligonu leotides were synthesized in order to amplify the CDMA encoding the peptide se-

SEG ID NO:3

T383B

2. CAGAGAATTCGTCGACA(AG)TC[N]GT[AG]TT[TC]TT

[YC]CY 3. ZEG ID NO:4

52 TODA=N snartw

protocol provided. First strand cDNA synthesis was performed as follows; Poly A* RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen and the

5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTT 3' SEQ ID NO:5, by incubating in a volume of 10 µl at , TLAT was annealled to 1 μg of adapter primer TA17,

 70°C for 5 min. followed by cooling to room temperature. To this reaction was added:

10X buffer (500 mM Tris-HCl pH 8.3, 750 mM KCl, 100 mM MgCl₂, 5 mM spermidine) M 2.5 14 0.E

TTQ Mm 001

10 mM each dATP, dGTP, dCTP, dTTP l4 2.5

lu 8.0 15 units RNasin M 2.5

40 mM Na pyrophosphate

M 2.5

and the reaction was incubated at 42°C for 1 hour, then diluted to 1 ml in 10 mM Tris-HCI I mM EDTA, ly G.F 15 units reverse transcriptase

.2.7 Hq

GΕ

OΖ

PCR Reactions:

Primary reaction (100 µl)

M 91 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP IL OF 10X buffer from Perkin Elmer Cetus GeneAmp kit

M S ANGo Jeso bnstte tenit

7 M 5.0 pMoles L42.2

S.S units Amplitaq DNA polymerase M S 50 pMoles T383' B

ld 2.78 lu 6.0

Reaction conditions, 40 cycles of 94°C, 1"; 50°C, 2"30"; 72°C, 2".

Prep scale secondary reaction:

10X buffer 100 h

1.25 mM each stock of dATP, dCTP, dGTP, and dTTP III 091

primary PCR reaction IL OF

500 pMoles L42.2 m oz

h 289 S5 units Amplitaq DNA polymerase Mβ 500 pMoles T383'B M OS

sequenced by the dideoxy chain termination method. ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and gested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The The PCR product was concentrated by Centricon 30 spin columns, purified on a 1% agarose gel, and di-Reaction conditions 94°C, 1"; 55°C, 2"; 72°C, 2"; 30 cycles.

PCR Amplification, Cloning and Sequencing of pW-3 01

subunit using the 3' RACE technique described by Frohman et al., PNAS 85: 8998-9002 (1988). 5'GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the COOH terminus of VEGF A CACTGTGAGC 3' SEQ ID NO:7. These primers were used in combination with oligoA 17 307 5' TITGTCGACTCAGAGGGGGGAAAAGC 3' SEQ ID NO:6 and oligo 289 5' TITGTCGACGAAAAT-Based on the sequence obtained from the p4238 clones, two specific PCR primers were synthesized; oligo

PCR reactions:

SO

68S ogilo seloMq 03 M S ANGo Je-20 bnette fenil lu 25.0 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP I4 81 10X buffer from Perkin Elmer Cetus GeneAmp kit M Or Primary reaction 100 µl

2.5 units Amplitaq DNA polymerase lų 2.0 52

58°C, 2"; 72°C, 40" followed by 40 cycles 94°C, 1"; 58°C, 2", 72°C, 2". Reaction conditions 94°C, 1°; 58°C, 2°; 72°C, 2°; 10 cycles then add 50 pMoles A17, then 1 cycle of 94°C, 1°; MS1.78

Prep Scale secondary reaction:

method.

300 pMoles oligo A17 12 M 300 pMoles oligo 307 12 m primary PCR reaction 74 hl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP lq 801 10X buffer III 09

Reaction conditions 94°C, 1"; 58°C, 2"; 72°C, 2"; 30 cycles.

assiemylog AMO pstilgmA stinu 31 Щε

1418E

PCR Amplification, Cloning and Sequencing of p5-15

SYTTTETCEACACACACAGGACGGCTTGAAG 3' SEQ ID NO:9 and oligo 74 5' Based on the sequence of p4238 clones, two specific PCR primers were synthesized; oligo 113

the cDNA encoding the amino terminus of VEGF A subunit using the 5' RACE technique described by Frohman These primers were used in combination with oligo A17 5' GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify TITETCEACATACTCCTGGAAGATGTCC 3' SEQ ID NO"10.

et al., supra. Oligo 151 was synthesized in order to specifically prime VEGF A subunit cDNA from GS-9L RNA.

blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform E. coli XL-1 The PCR product was pwified on a 1% agarose gel and digested with restriction endonuclease Sa11. The

provided. First strand cDNA synthesis was performed as follows; RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen using the protocol CTTCATCATTGCAGC 3' SEQ ID NO:11.

5' followed by cooling to room temperature. To this reaction was added: One µg of GS9L RNA was annealled to 1 µg of oligo 151 by incubating in a volume of 6 µl at 70°C for

FP 0 206 477 A1

reaction was incubated at 42°C for 1 hour. Excess oligo151 was removed by Centricon 100 spin col-	euT	
20 units diluted reverse transcriptisse	lu 2.9	
40 mM Na pyrophosphate	lu 2.5	ç
S5 units RNasin	lu 3.0	
10 mM each dATP, dGTP, dCTP. dTTP	ել Շ.Տ	
TTG Mm 01	ել Շ.Տ	
10X buffer (500mM Tris-HCl, pH 8.3, 750 mM KCl, 100 mM MgCl ₂ , 5 mM spermidine)	lų č. ř	

was diluted to a final volume of 150 µl in 10 mm Tris-HCI, 1 mM EDTA, pH 7.5. umns and the 5' end of the cDNA was tailed by the addition of dATP and terminal transferase. The tailed cDNA

PCR Reactions:

ction conditions; 1 cycle 94°C 1°; 50°C 2°; 72°C 40° then 40 cycles of 94°C 1°; 50°C 1°30°; 72°C 2°	Rea	
Water (1905) 110 200 111 200 12 12 12 12 12 12 12 12 12 12 12 12 12	ել ՇԴ.82	
essznemylog AVO ptildmA stinu ZS.f	ա 62.0	50
TIAT ogilo saloMq 01	lųΓ	
ZIA ogilo saloMq ZS	իկ Ր	
St poligo oligo 113	ելի	
first strand GS-9L cDNA prime with oligo 151 and tailed	M 3	
1.25 mM each stock of dATP, dCTP, dGTP, and dTTP	Ιц 8	S1
10X buffer from Perkin Elmer Cetus GeneAmp Kit	lų č	
(ly 03) reaction (50 ly)	nsmin9	

Prep scale secondary reaction:

1etsw	ከ የ የ የ	
15 units Amplitaq DNA polymerase	lų ε	
300 pMoles oligo ATA	12 m	90
300 pMoles oligo 24	14 St	
primary PCR reaction	l4 θ	
1.25 mM each stock of dATP, dCTP, dGTP, and dTTP	m 96	
10X buffer	lų 03	
		52

E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain lease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform The PCR product was concentrated by Centricon 100 spin columns, and digested with restriction endonuc-Reaction conditions 94°C, 11; 55°C, 21; 72°C, 21 30 cycles.

Cloning and sequencing of alternative forms of VEGF A cDNA termination method. The base sequence is shown in Fig. 5.

tiple cDNAs encoding alternative forms of the VEGF A subunit. TTTGTCGACGGTGAGAGGTCTAGTTC 3' SEQ ID NO:13. These primers were used together to amplify multhesized; oligo 5' C 5' TTTGTCGACAACCATGAACTTTCTGC 3' SEQ ID NO:12 and oligo 181 5' Based on the sequence obtained from the p5-15 and pW-3 dones, two specific PCR primers were syn-

Preparative PCR Reaction:

07

	ա Յ.Ն	Water
	ե, 5 թվ	15 units Amplitad DNA polymerase
	lų Or	300pMoles oligo 181
	₩ Or	300pMoles oligo 5°C
0	lų Or	first strand GS-9L cDNA
	lų 08	1.25mM each stock of dATP, dCTP, dGTP, and dTTP
	14 03	10X buffer

The ligation mix was used to transform E.coli XL-1 blue. Plasmid DNA was isolated from white transformants cipitated by ethanol, and digested with restriction endonuclease Sal I, and ligated into SalI cut pGEM3Zf(+). The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, pre-Reaction conditions 94°C, 1"; 58°C, 2"; 72°C, 3"; 40 cydes.

A subunit with the conversion of Asn¹⁴⁰ to Lys¹⁴⁰, Figure 6. the second and third base of the Asn³40 codon. This done thus encodes the 214 amino acid form of the ∀EGF begin until Cys166, this form also finishes at Arg199, Figure 4. Clone #16 has a 72 base pair insertion between amino acid secreted form of VEGF A subunit runs from Ala²⁷ to Asn 140, which becomes Lys 140 and does not This done thus encodes a 146 as form of the VEGF A subunit with the conversion of Asn 140 to Lys 140. The 120 a 135 base pair deletion between the second base of the Asn¹40 codon and the third base of the Arg¹84 codon. form of VEGF A subunit is that amino acid sequence running continuously from Ala²⁷ to Arg¹⁹⁰. Clone#14 has coded the 190 amino acid form of VEGF A subunit identical to that shown in Fig. 1. The 164 amino acid secreted and sequenced by the dideoxy chain termination method. Three sets of dones were identified. Clone#12 en-

EXAMPLE 10

Cloning and Sequencing of the VEGF B Subunit

PCR Amplification, Cloning and Sequencing of pYG 91

quences of VEGF B on Lys C fragment L50. These oligonucleotides were: Two degenerate oligonucleotides were synthesized in order to amplify the cDNA encoding the peptide se-

GC 5' TITGTCGACTC[AG]TC[AG]TT[AG]CA[AG]CA[N]CC 3' SEQ ID NO:15 where N=ACGT YI S' TTTGTCGACATA[TCJAT[TCA]GC[N]GA[TC]GA[AG]C 3' SEQ ID NO:14

provided. First strand cDNA synthesis was performed as follows; ANA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen and the protocol

5'GACTCGAGTCGACATCGATTTTTTTTTTTTT 3' SEQ ID NO: 5, by incubating in a volume of 10 µl at 1 trg of GS-9L poly A+RNA was annealled to 1 trg of adapter primer TA17.

₩ 0.£ $70^{\circ}\mathrm{c}$ for 5 min. followed by cooling to room temperature. To this reaction was added:

10X buffer (500 mM Tris-Hd, pH 8.3, 750 mM KCl, 100 mM MgCl $_{\rm 2}$, 5mM spermidine) 14 G.S

TTG Mm 001 M 2.5

10 mM each dATP, dGTP, dCTP, dTTP M 2.5

L.5 H 15 units RNasin M 9.0 30

15 units reverse transcriptase 40 mM Na pyrophosphate

and the reaction was incubated at 42°C for 1 hour, then diluted to 1 ml in 10 mM Tris-HCl, I mM EDTA,

.č.7 Hq

35

PCR Reactions:

Primary reaction (50µl)

lų r 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP μβ 10X buffer from Perkin Elmer Cetus GeneAmp kit μç

ANGo Je-80 bnette ferif

IY ogilo saloMq 03 μL

lщ 27.££ 1.25 units Amplitaq DNA polymerase M 22.0 50 pMoles oligo GC μt

Reaction conditions, 40 cycles of 94°C, 1"; 50°C, 2"; 72°C, 2".

Prep scale reaction:

1.25mM each stock of dATP, dCTP, dGTP, and dTTP m 96 10X buffer III 09

ANGo Jeed bnatte frain 12 M

ա ՀՐ IY ogilo səloMq003 12 M

15 units Amplitaq DNA polymerase щε 99 500pMoles oligo GC

402 M

The PCR product was concentrated by Centricon 30 spin columns and digested with restriction endonuc-Reaction conditions 94°C, 1°; 50°C, 2°; 72°C, 2° 40 cycles.

termination method. E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain lease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform

PCR Amplification, Cloning and Sequencing of p3V2

Based on the sequence obtained from the pYG clones, a specific PCR primer was synthesized; oligo HP

This primer was used in combination with oligo A17 5' 5' TTTGTCGACACACCCTAATGAAGTGTC 3' SEQ ID NO:16.

subunit using the 3' RACE technique described by Frohman et al., PNAS 85: 8998-9002 (1988). GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the COOH terminus of the VEGF B

Preparative PCR reaction:

ANGo Jeed bnsva tenit 12 M Щ 09 10X buffer from Perkin Elmer Cetus Gene Amp Kit

1.25 mM each of dATP, dCTP, dGTP, dTTP m 96

14 St TIA ogilo seloMq 008

300 pMoles oligo HP

essiemylog ANG petilgmA stinuct 12 M

Щε

M 504

Reaction conditions 1 cycle of 94°C, 1"; 58°C, 2"; 72°C, 2"; followed by 40 cycles 94°C, 1", 58°C, 2" and

72°C, 2°.

enced by the dideoxy chain termination method. mix was used to transform E. coli. XL-1 blue. Plasmid DNA was isolated from white transformants and sequwith restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation The PCR product was concentrated by Centricon 30 spin columns, precipitated with ethanol and digested

PCR Amplification, Cloning and Sequencing of p5V2

5' TTTGTCGACAACAGCGACTCAGAAGG 3' SEQ ID NO: 17 and oligoVS' 5' TTTGTCGACACTGAATATAT-Based on the sequence of pYG clones, two specific PcR primers were synthesized; oligoVL'

GAGACAC 3' SEQ ID NO:18. These primers were used in combination with oligo A17

B subunit using the 5' RACE technique described by Frohman et al., supra. Oligo 151 was synthesized in order 5' GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the amino terminus of the VEGF

Poly A+RNA was isolated from GS9L cells using the Fast Track RNA isolation kit from Invitrogen using the to prime cDNA from GS-9L RNA Oligo 151 is 5' CTTCATCATTGCAGCAGC 3' SEQ ID NO:11.

protocol provided. First strand cDNA synthesis was performed as follows:

incubating in a volume of 6 µl at 70°C for 5' followed by cooling One µg of GS9L RNA was annealled to 1 µg of oligo 151 by

to room temperature. To this reaction was added:

M 2.5 TTG Mm Of 10X buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 100 mM MgCl₂, 5mM spermidine) In C.1

10 mM each dATP, dGTP, dCTP, dTTP l4 2.5

Щ 9.0 25 units RNasin

M 2.5 40 mM Na pyrophosphate

The reaction was incubated at 42°c for 1 hour. 20 units diluted reverse transcriptase M 6.9

mM Tris-HCI, 1 mM EDTA, pH 7.5 of it in 150 pt 150 pt 150 pt 150 pt 151 pt 151 pt 151 pt 151 pt 150 pt Excess oligo 151 was removed by Centricon 100 spin columns and the 5' end of the cDNA was tailed by

PCR Reactions:

07

Primary reaction (50 µl) 99

M 8 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP Щδ 10X buffer from Perkin Elmer Cetus GeneAmp Kit

μç first strand GS9L cDNA primed with oligo151 and tailed

ld 27.82 esseremylog AMQ pillgmA stinu 32.1 **M 22.0** 10 pMoles oligo TA17 MI TIA ogilo seloMq 35 lų r 25 pMoles oligo VL' lų r

Reaction conditions; 1 cycle 94°C,1°; 58°C, 2°; 72°C, 40° then 40 cycles of 94°C, 1°; 58°C, 2°; 72°C, 2°.

Prep scale secondary reaction

S5 units Amplitaq DNA polymerase lt c 71A ogilo seloMq 00£ In OS 500 pMoles oligo VS' **50 m** primary PCR reaction IN OF 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP 14 09 L 10X buffer 14 00 F

Reaction conditions 94°C, 1°; 58°C, 2°; 72°C, 2° 30 cycles. M 289

XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination Nu-Sieve Agarose gel then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform E. coli cipitated by ethanol, and digested with restriction endonuclease Sall. The Sall fragment was purified on 4% 50 The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, pre-

PCR Amplification, Cloning and Sequencing of pCV2 and pCV2.1

Based on the sequences of the p3V2 and p5CV2 clones, two specific PCR primers were synthesized; oligo S'CV2.1

TITETCEAC[N][N]CTAATAAATAGAGG 3' SEQ ID NO:20. 5' TTTGTCGAC[N][N]GCAGGTCCTAGCTG 3' SEQ ID NO;19 and oligo 3'CV2 5'

These primers were used together to amplify the cDNA encoding the VEGF B subunit.

Preparative PCR Reaction:

52

10units Amplitaq DNA polymerase Щ 2 200 byoies 3,CAS Щ 8 200 pMoles 5'CV2.1 Щ 8 ANGo Je-20 bnette fenit M 8 1.25 mM each dATP, dTTP, dGTP, dCTP l4 49 32 10X buffer M 04

M 072 00

158 amino acid sequence and the other encoded a 138 amino acid sequence, see Figures 7 and 8. and sequenced by the dideoxy chain termination method. Two sets of dones were identified, one encoded a The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated form white transformants cipitated by ethanol, and digested with restriction endonuclease Sal 1, and ligated into Sal I cut pGEM3Zf(+). The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, pre-Reaction conditions: 94°C, 1', 58°C, 2', 72°C, 2'; 40 cycles.

cDNA Cloning of VEGF B Subunit

First Strand Synthesis from a cDNA done isolated from a cDNA library constructed from GS-9L polyA+ RNA. The DMA and protein sequences for the amino terminus of the signal peptide of VEGF B was determined 09

Anneal 15.6µl (5ug) 6S-9L polyA+ RNA and 2.5µl (2.5ug) oligo dT-Xbal primer by heating to 70° C 5°

10X buffer (500 mM Tris-HCl, pH 8.3 (42° C), 750 mM KCl, 100 mM MgCl₂, 5mM spermidine TTO Mm001 MG.G MG.2 slow cool to room temperature. Add the following:

nissNA (atinučč) M4.1 10 mM each dATP, dTTP, dGTP MG.B

Incubate at 42° C 60°. MG.E1 (55units) reverse transcriptase MG.B 40mM NaPPi

Second Strand Synthesis:

first strand reaction Assemble reaction mix

10X buffer (500 mM Tris-HCi, pH7.2, 850 mM KCL, 30 mM MgCl₂ 1mg/ml BSA, 100 mM (NH₄)₂SO₄ M 05

14 SS

lu C.T TTG Mm 001

ld 6.8 (65unita) E. coli DNA Polymerasel M 52 **GAN Mmt**

M 2.5 (2.5units) E. coli DNA Ligase

M 2.5 (2 units) E. coli RNase H

of 10 µ 02 m scetate and 3 volumes of ethanol, collect precipitate and resuspend in 20 µ of 10 at 37° C for 10', add 25 µl 0.2M EDTA an extract with phenol/chloroform, then precipitate by the addition of 0.5 Incubate at 14° C for 2h and then incubate 70° C for 10'. Add 1 µl (10 units) T4 DNA Polymerase, incubate 132 m

MM Tris-HCI, pH 7.5, 1mM EDTA.

cDNA Library Construction

addition of EcoR1 linkers and digestion with EcoR1 and Xbal. A cDNA library was amplified from ~50, 000 The above cDNA was ligated into EcoR1\ Xbal digested LambdaGEM-4 (Promega Biochemicals) after the

independent dones.

The above cDNA library was screened by placque hybridization using pCV2 as a probe. Hybridization con-Isolation of Rat VEGF B cDNA Clone

5XSSC (1XSSC is 0.15M sodium chloride, 0.015M sodium citrate, ditions were as follows:

50% Formamide

5X Denhardt's Solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin)

Filters were washed 3 times in 2XSSC, 0.1% SDS at room temerature for 5', then 1 time in 1XSSC, 0.1% 0.15 mg/ml salmon sperm DNA hybridize overnight at 42° C.

isolated from white transformants and sequenced by the dideoxy chain termination method. The cDNA sequinto Xbal digested pGEM3Zf(+). The ligation mix was used to transform E.coii XL-1 blue. Plasmid DNA was The DNA from phage #202 was digested with restriction endonuclease Spel and the 11kb band ligated SDS at 50C for 30°. Positive dones were identified by autoradiography.

protein starts at Ala²⁴ and continues to Arg¹³⁸. The entire nucleotide and amino acid sequence of the 158 amino The entire nucleotide and amino acid sequence of the 136 amino acid form is shown in Fig. 7. The secreted ence and predicted amino acid sequence of the signal peptide are shown in Figures 7 and 8.

scid form is shown in Figures 8. The secreted protein starts at Ala²⁴ and continues to Leu¹⁵⁸.

EXAMPLE 11

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01

Cloning and sequencing VEGF C Subunit

PCR Amplification, Cloning and Sequencing of pFSEM'

der to amplify VEGF cDNAs from the human medulloblastoms line TE-671, ATCC HTB (McAllister et al., Int. Two degenerate oligonucleotides were synthesized based on the sequence of rat VEGF B monomer in or-

FS 5'TTTGTCGACA TTC AGT CC(N) TC(N) TG(TC) GT 3' SEQ ID NO:21 J. Cancer 20:206-212 [1977]). These oligonucleotides were:

EM' 5' TTTGTCGACA CTG AGA GAA (N)GT CAT (CT)TC 3'

SEO ID NO:22

M61

the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from 99 Poly A+ RNA was isolated from IE-671 cells using the Fast Track RNA isolation kit from Invitrogen and Where N= AGCT

ANA +Aylog 178-3T to 641 lu, r Invitrogen;

water

5X RT buffer MOT RNase Inhibitor M2.5 random primer M2.5 0.7M B-mercaptoethanol M2S.8 HOPMAM Mm001

reverse transcriptase 12.5units 1.25µ L.5_M SamM dNTPs

The reaction was incubated for 60' at 42°C, then 3' at 95°C, placed on ice, then an additional 1.25ul reverse

The above procedure was performed in duplicate and the cDNAs pooled to a final volume of 100ul. au80° at 42° C.

PCR Reactions:

1.25mM each of dATP, dCTP, dGTP, TTP M 91 10X buffer from Perkin Elmer Cetus GeneAmp kit M OF SI Primary reaction (100µ)

50 pmoles FS primer Щ 2 first strand TE-671 cDNA In Or

50 pmoles EM primer M 2

2.5 units Amplitaq DNA polymerase lų č.0 oz

M 2.65

Gel Purification Reaction conditions: 40 cycles of 90°C, 1"; 2" ramp to 45°C; 2" at 45°C; 2" at 72°C.

אס וון סל נוופ primary PCR reaction was purified on a 4% NuSieve agarose gel. The 180 base pair band was

excised from the gel. heated to 65°C for 5' and used directly as template for the secondary PCR reaction.

10X buffer from Perkin Elmer Cetus GeneAmp kit 20 M Secondary PCR reaction 200µl

32 pt

metted get slice μç 1.25mM each of dATP, dCTP, dGTP, TTP

100 pmoles FS primer M 4

100 pmoles EM' primer M 4

Sunits Amplitad DNA polymerase M L

134 M

Reaction conditions: 35 cycles of 94°C, 1"; 50°C, 2"; 72°C, 2"

XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform E. coli The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall.

PCR Amplification, Cloning and Sequencing of p3'.19

terminus of the VEGF C subunit using the 3' RACE technique described by Frohman et al., PNAS 85:8998-9002 nation with oligo A17,5' GACTCGAGTCGACATCG 3' SEQ ID NO:24, to amplify the cDNA encoding the COOH oligo LH 5' TTTGTCGACA CTG CAC TGT GTG CCG GTG 3' SEQ ID NO:23. This primer was used in combi-Based on the sequence obtained from the pFSEM done, a specific PCR primer was synthesized;

the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and

TAIT 5' GACTCGAGTCGACATCGATTTTTTTTTTTTT 3' SEQ ID NO:5 Invitrogen and the TA17 adapter primer.

ANA +Ayloq ITS-ST to gulf lu 8.0

Water ILI 7.02

L 32.9 HOgMaM Mm 001 μç

RNase Inhibitor M 6.5 TIAT 19ming gu 88.0 M 0.1 0.7 M B-mercaptoethanol

25mM dNTPs M 2.5 5X RT buffer IL OF

92

32

30

transcriptisse was added and the reaction incubated an additional 60' at 42°C. The reaction was incubated for 60' at 42°C, then 3' at 95°C, placed on ice, then an additional 1.25ul reverse reverse transcriptase 12.5 units

3, RACE PCR

M 2 50 pmoles LH primer 10 Trat attand TE-671 cDNA primed with Trat M 02 1.25mM each of dATP, dCTP, dGTP, TTP 14 SE 20 M 10 X buffer from Perkin Elmer Cetus GeneAmp kit

50 pmoies A17 primer 14 S

5 units Amplitaq DNA polymerase Щ 0.1

123 H

Reaction conditions: 40 cycles of 94C, 1"; 2" at 58°C; 3" at 72°C.

XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform E. coli The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall.

PCR Amplification, Cloning and Sequencing of p5.16

oligo A17, 5' GACTCGAGTCGACATCG 3' SEQ ID NO:8, and oligo TA17 ATC GCC GCA GCC GGT 3' SEQ ID NO:25. These primers were used in combination with Oligo VE' 5' TTTGTCGACA AC ATT GGC CGT CTC CAC C 3' SEQ ID NO:24, and oligo TG' 5' TTTGTCGACA Based on the sequence obtained from the pFSEM' done, two specific PCR primers were synthesized;

terminus of the VEGF C subunit using the 5' RACE technique described by Frohman et al., PNAS 85: 8998-9002 5'GACTCGACTCGACATCGACTTTTTTTTTTT3' SEQ ID NO:5 to amplify the cDNA encoding the amino

Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and

the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from

ANA +Aylog 178-3T to gul 1 M 0.1 Invitrogen and the VE' primer:

교 22.02

0.7 M B-mercaptoethanol ել 62.8 μlG HOgMeM Mm 00t

M Or 5X RT buffer M 2.5 RNase Inhibitor 1.0 µg primer VE' l4 0. ₽

25 mM dNTPs l4 6.5

transferace. The tailed cDNA was diluted to a final volume of 200 ul in 10mM Tris-HCl, 1mM EDTA, pH 7.5. by a Centricon 100 spin column and the 5' end of the cDNA was tailed by the addition of dATP and terminal transcriptage was added and the reaction incubated an additional 60' at 42°C. Excess oligo VE' was removed The reaction was incubated for 60'st 42°C, then 3' at 95°C, placed on ice, then an additional 1.25ul reverse AMV reverse transcriptase (Promega) 10units

S' RACE PCR 5 X 100ul

09

M Or first strand TE-671 cDNA primed with VE' l4 91 1.25mM each of dATP. dCTP, dGTP, TTP 10X buffer from Perkin Elmer Cetus GeneAmp kit M Of

50 pmoles A17 primer lu S 50 pmoles TG' primer

M 2 19ming TIAT selomg 0S ld S

22 2.5 units AMplitaq DNA polymerase ₩ **2.0**

The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform E. coli The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall. Reaction conditions: 40 cycles of 94°C, 11; 2' ramp to 58°C; 2' at 58°C; 2' at 72°C.

method. The combined sequences form plasmids pFSEM', p3'19 and p5'16 are shown in Figure 9. XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination

PCR Amplificpotion, Cloning and Sequencing of phVC16 and phVC2

coding the complete VEGF C subunit. ACT GAA GAG TGT GAC GG 3' SEQ ID NO:27. These primers were used together to amplify the cDNA enoligo 5' GCVB 5' TTTGTCGAC TGG CTC TGG ACG TCT GAG 3' SEQ ID NO:26 and oligo 3'VC 5' TTTGTCGAC Based on the sequences of the p5'. 16 and p3'. 19 clones, two specific PCR primers were synthesized;

the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and

Jeming Tb ogilo luc.S lonsitieotosom-6 M T.0 M22.9 100 mM MeMgOH μiς Water M2.91 ANA +Ayloq LT8-ST to gult lщ8.0

25 mM dNTPs LIG.S 5X RT buffer 10pl RNase Inhibitor M2.5

reverse transcriptase 12.5units Mes.1

PCR Reaction 200 ul transcriptase was added and the reaction incubated an additional 60' at 42C. The reaction was incubated for 60' at 42C, then 3' at 95C, placed on ice, then an additional 1.25ul reverse

10X buffer from Perkin Elmer Cetus GeneAmp kit ZOM

50 pmoles 5' GCVB primer μļρ The ogilo with bemine ANDs 178-3T basts tank 1402 1.25mM each of dATP. dCTP, dGTP, TTP 324

50 pmoles 3'VC primer 414

5 units Amplitad DNA polymerase MI

merr

method. In the sequences of clones phVC16 and phVC2 base 463 (Fig. 9) was changed from a T to a C elimi-XL-1 blue. Plasmid DMA was isolated from white transformants and sequenced by the dideoxy chain termination The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform E. coli The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall. Reaction conditions: 40 cycles of 94°C, 1';; 2' at 50°C; 2'at 72°C.

lowing amino acid Lys 154. The nucleotide sequence and the deduced amino acid sequence of this addition nating the translational atop codon following amino acid 154; this results in the addition of 16 amino acids fol-

Cln Arg Pro Thr Asp Cys His Leu Cys Gly Asp Ala Val TTO TOO TAD DOD DOT DID DAD ADA DOD ADA DAD

SEQ ID NO-29 OLI Pro Arg Arg AAT DEA DED DOD

deletion of Gln 25. In addition done phVC16 contains a 3 base pair deletion (Figure 9, nucleotide residues 73-75) resulting in the 99

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. 0 \$			
SP			•
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SS			
50			
ē1		(2)	••••
01		(I) SEŌO (B) (C)	ENCE CHARACTERISTICS: LENGTH: 14 amino acids TYPE: amino acid STRANDEDNESS: Not Applicable TOPOLOGY: linear
ç	(S)		ON FOR SEQ ID NO:1:

(2) INFORMATION FOR SEQ ID NO.5: (2) INFORMATION FOR SEQ ID NO.5: (3) INFORMATION FOR SEQ ID NO.5: (4) SEQUENCE CHARACTERISTICS: (5) INFORMATION FOR SEQ ID NO.6: (6) TENGTH: 19 Small Seq ID NO.5: (7) SEQUENCE CHARACTERISTICS: (8) TENGTH: 35 Dase pairs (1) SEQUENCE CHARACTERISTICS: (2) INFORMATION FOR SEQ ID NO.5: (3) SEQUENCE CHARACTERISTICS: (4) LENGTH: 35 Dase pairs (5) INFORMATION FOR SEQ ID NO.5: (6) STRANDEDNESS: Single (7) SEQUENCE CHARACTERISTICS: (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: Single (3) TOPOLOGY: Linear (4) LENGTH: 35 Dase pairs (5) SEQUENCE CHARACTERISTICS: (6) TOPOLOGY: Linear (7) SEQUENCE CHARACTERISTICS: (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: Single (3) SEQUENCE CHARACTERISTICS: (4) LENGTH: 35 Dase pairs (5) STRANDEDNESS: Single (6) STRANDEDNESS: Single (7) SEQUENCE CHARACTERISTICS: (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: Single (3) SEQUENCE CHARACTERISTICS: (4) LENGTH: 35 Dase pairs (5) STRANDEDNESS: Single (6) STRANDEDNESS: Single (7) SEQUENCE CHARACTERISTICS: (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: Single (3) SEQUENCE CHARACTERISTICS: (4) SEQUENCE CHARACTERISTICS: (5) STRANDEDNESS: Single (6) STRANDEDNESS: Single (7) SEQUENCE CHARACTERISTICS: (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: Single (3) STRANDEDNESS: SINGLE (4) SEQUENCE CHARACTERISTICS: (5) STRANDEDNESS: Single (6) STRANDEDNESS: Single (7) STRANDEDNESS: Single (8) TYPE: NUCLEIC (9) STRANDEDNESS: Single (10) SEQUENCE CHARACTERISTICS: (11) SEQUENCE CHARACTERISTICS: (12) SEQUENCE CHARACTERISTICS: (3) STRANDENESS: SINGLE (4) STRANDENESS: SINGLE (5) STRANDENESS: SINGLE (6) STRANDENESS: SINGLE (7) STRANDENESS: SINGLE (8)	· · · · · · · · · · · · · · · · · · ·	
4 Val (2) INFORMATION FOR SEQ ID NO:5: (2) INFORMATION FOR SEQ ID NO:3: (3) INFORMATION FOR SEQ ID NO:3: (4) SEQUENCE CHARACTERISTICS: (5) INFORMATION FOR SEQ ID NO:4: (6) STRANDEDESS: SINGLE (7) SEQUENCE CHARACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDESS: SINGLE (3) INFORMATION FOR SEQ ID NO:4: (4) SEQUENCE CHARACTERISTICS: (5) STRANDEDESS: SINGLE (6) STRANDEDESS: SINGLE (7) SEQUENCE CHARACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) SEQUENCE CHARACTERISTICS: (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) SEQUENCE CHARACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) SEQUENCE CHARACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) SEQUENCE CHARACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) STREAMS STRANDEDNESS: SINGLE (2) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) STRANDEDNESS: SINGLE (8) TYPE: MUCHACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: LINEAR (10) STRANDENESS: SINGLE (11) SEQUENCE CHARACTERISTICS: (12) STRANDENESS: SINGLE (4) STRANDENESS: SINGLE (5) STRANDENESS: SINGLE (6) STRANDENESS: SINGLE (7) STRANDENESS: SINGLE (8) TYPE: MUCHACTERISTICS: (8) TYPE: MUCHACTERISTICS: (9) TOPOLOGY: LINEAR (10) STRANDENESS: SINGLE (10) STRANDENESS: SINGLE (11) STRANDENESS: SINGLE (12) STRANDENESS: SINGLE (13) STRANDENESS: SINGLE (14) STRANDENESS: SINGLE (15)	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid	<i>0</i> ⊊
(C) STRANDEDNESS: SINGLE (D) TOPOLOCY: Linear (A) SEQUENCE CHARACTERISTICS: (B) TYPE: NUCLEIC SINGLE (C) STRANDEDNESS: SINGLE (D) TOPOLOCY: Linear (A) SEQUENCE CHARACTERISTICS: (A) SEQUENCE CHARACTERISTICS: (B) TYPE: NUCLEIC STRANDEDNESS: SINGLE (B) TYPE: NUCLEIC STRANDEDNESS: SINGLE (B) STRANDEDNESS: SINGLE (C) STRANDEDNESS: SINGLE (A) SEQUENCE CHARACTERISTICS: (B) STRANDEDNESS: SINGLE (C) STRANDEDNESS: SINGLE (D) TOPOLOCY: LINEAR (D)	(xt) SEGNENCE DESCRIBLION: SEG ID NO:2:	S7*
val Plan Tint Gid Giv Gid Gid Lys Ala His Giu Val (2) INFORMATION FOR SEQ ID NO:2: (2) INFORMATION FOR SEQ ID NO:4: (3) INFORMATION FOR SEQ ID NO:4: (4) SEQUENCE CHARACTERISTICS: (5) INFORMATION FOR SEQ ID NO:4: (6) TENGTH: 19 amino acida (7) SEQUENCE CHARACTERISTICS: (8) TYPE: mucleic acid (9) TENGTH: 26 base pairs (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: single (3) INFORMATION FOR SEQ ID NO:4: (4) LENGTH: 32 base pairs (5) STRANDEDNESS: single (6) STRANDEDNESS: single (7) SEQUENCE CHARACTERISTICS: (8) TYPE: mucleic acid (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: single (3) INFORMATION FOR SEQ ID NO:4: (4) LENGTH: 32 base pairs (5) STRANDEDNESS: single (6) STRANDEDNESS: single (7) SEQUENCE CHARACTERISTICS: (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: single (3) STRANDEDNESS: single (4) LENGTH: 32 base pairs (5) STRANDEDNESS: single (6) STRANDEDNESS: single (7) STRANDENESS: single (8) TYPE: nucleic acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: single (3) STRANDENESS: single (4) STRANDENESS: single (5) STRANDENESS: single (6) STRANDENESS: single (7) STRANDENESS: single (8) TYPE: mucleic acid (8) TYPE: mucleic acid (9) TYPE: mucleic acid (10) TOPOLOGY: Linear (11) SEQUENCE CHARACTERISTICS: (12) STRANDENESS: single (3) STRANDENESS: single (4) STRANDENESS: single (5) STRANDENESS: single (6) STRANDENESS: single (7) STRANDENESS: single (8) TYPE: mucleic acid (9) TYPE: mucleic acid (10) TOPOLOGY: Linear (11) SEQUENCE CHARACTERISTICS: (12) STRANDENESS: single (3) STRANDENESS: single (4) STRANDENESS: single (5) STRANDENESS: SINGLE (6) STRANDENESS: SINGLE (7) STRANDENESS: SINGLE (8) TYPE: MUCLEIC STRANDENESS: SINGLE (8) TYPE: MUCLEIC STRANDENESS: SINGLE (9) TYPE: MUCLEIC STRANDENESS: SINGLE (10) TYPE: MUCLEIC STRANDENESS: SINGLE (11) TYPE: MUCLEIC STRANDENESS: SINGLE (12) STRANDENESS: SINGLE (13) STRANDENE	(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 35 Dase pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single	O V
val (2) INFORMATION FOR SEQ ID NO:4: (2) INFORMATION FOR SEQ ID NO:4: (2) INFORMATION FOR SEQ ID NO:4: (3) INFORMATION FOR SEQ ID NO:3: (4) SEQUENCE CHARACTERISTICS: (5) INFORMATION FOR SEQ ID NO:3: (6) STRANDEDNESS: single (7) SEQUENCE CHARACTERISTICS: (8) TOPOLOGY: linear (9) TOPOLOGY: linear (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: single (3) INFORMATION FOR SEQ ID NO:3: (4) LENGTH: 30 base pairs (5) INFORMATION FOR SEQ ID NO:3: (6) STRANDEDNESS: single (7) SEQUENCE CHARACTERISTICS: (8) TOPOLOGY: linear (9) TOPOLOGY: linear (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) INFORMATION FOR SEQ ID NO:4: (3) SEQUENCE CHARACTERISTICS: (4) SEQUENCE CHARACTERISTICS: (5) INFORMATION FOR SEQ ID NO:4: (6) STRANDEDNESS: Single (7) SEQUENCE CHARACTERISTICS: (8) SEQUENCE CHARACTERISTICS: (9) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: Single (3) SEQUENCE CHARACTERISTICS: (4) SEQUENCE CHARACTERISTICS: (5) STRANDEDNESS: Single (6) STRANDEDNESS: Single (7) SEQUENCE CHARACTERISTICS: (8) STRANDEDNESS: SINGLE (9) STRANDEDNESS: SINGLE (10) STRANDEDNESS: SINGLE (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) SEQUENCE CHARACTERISTICS: (6) STRANDEDNESS: SINGLE (7) STRANDEDNESS: SINGLE (8) STRANDEDNESS: SINGLE (9) STRANDEDNESS: SINGLE (10) STRANDEDNESS: SINGLE (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) STRANDEDNESS: SINGLE (8) STRANDEDNESS: SINGLE (9) STRANDEDNESS: SINGLE (10) STRANDEDNESS: SINGLE (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (13) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) STRANDEDNESS: SINGLE (8) STRANDEDNESS: SINGLE (9) STRANDEDNESS: SINGLE (10) STRANDEDNESS: SINGLE (11) STRANDEDNESS: SINGLE (12) STRANDEDNESS: SINGLE (13) STRANDEDNESS: SINGLE (14) STRANDEDNESS: SINGLE	(C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	32
2 Val PLO THE TIME GIU GIN US ALA HIS GIU VAL (2) INFORMATION FOR SEQ ID NO:3: (2) INFORMATION FOR SEQ ID NO:3: (3) INFORMATION FOR SEQ ID NO:3: (4) SEQUENCE CHARACTERISTICS: (5) INFORMATION FOR SEQ ID NO:3: (6) TOPOLOGY: Linear (7) SEQUENCE CHARACTERISTICS: (8) TYPE: amino acid (9) TOPOLOGY: Linear (1) SEQUENCE CHARACTERISTICS: (2) STRANDEDNESS: SINGLE GIU VAL (3) SEQUENCE CHARACTERISTICS: (4) LENGTH: 19 amino acida (5) STRANDEDNESS: NOC Applicable (6) TOPOLOGY: Linear (7) SEQUENCE CHARACTERISTICS: (8) TYPE: amino acida (9) TOPOLOGY: Linear (10) TOPOLOGY: Linear (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (3) SEQUENCE CHARACTERISTICS: (4) SEQUENCE CHARACTERISTICS: (5) STRANDEDNESS: SINGLE (6) TOPOLOGY: Linear (7) STRANDEDNESS: SINGLE (8) TOPOLOGY: Linear (8) TOPOLOGY: Linear (9) TOPOLOGY: Linear (10) TOPOLOGY: Linear (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) SEQUENCE CHARACTERISTICS: (6) TOPOLOGY: Linear (7) SEQUENCE CHARACTERISTICS: (8) TOPOLOGY: Linear (9) TOPOLOGY: Linear (10) TOPOLOGY: Linear (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (3) STRANDEDNESS: SINGLE (4) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) TOPOLOGY: Linear (7) SEQUENCE CHARACTERISTICS: (8) TYPE: Amino SCICA (9) TOPOLOGY: Linear (10) TOPOLOGY: Linear (11) SEQUENCE CHARACTERISTICS: (12) STRANDEDNESS: SINGLE (13) STRANDEDNESS: SINGLE (4) STRANDEDNESS: SINGLE (5) STRANDEDNESS: SINGLE (6) STRANDEDNESS: SINGLE (7) STRANDEDNESS: SINGLE (8) TOPOLOGY: Linear (8) TOPOLOGY: Linear (8) TOPOLOGY: Linear (9) TOPOLOGY: Linear (12) STRANDEDNESS: SINGLE (13) STRANDEDNESS: SINGLE (14) STRANDEDNESS: SINGLE (15) STRANDEDNESS: SINGLE (16) STRANDEDNESS: SINGLE (17) STRANDEDNESS: SINGLE (18) STRANDEDNESS: SIN	(2) INFORMATION FOR SEQ ID NO:4: (1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 DASE pairs	οε
val Pro Inf The Giu Giu Gin Lys Ala His Giu Val (2) INFORMATION FOR SEQ ID NO:3: (2) INFORMATION FOR SEQ ID NO:2: (3) INFORMATION FOR SEQ ID NO:2: (4) SEQUENCE DESCRIPTION: SEQ ID NO:2: (5) SEQUENCE DESCRIPTION: SEQ ID NO:2: (6) TYPE: amino acid (7) SEQUENCE DESCRIPTION: (8) TYPE: amino acid (8) TYPE: amino acid (9) TOPOLOGY: linear (8) TYPE: amino acid (8) TYPE: amino acid (9) TOPOLOGY: linear (8) TYPE: amino acid (9) TOPOLOGY: linear (10) TOPOLOGY: linear (10) TOPOLOGY: linear (11) SEQUENCE DESCRIPTION: SEQ ID NO:2: (12) SEQUENCE DESCRIPTION: SEQ ID NO:3: (8) TYPE: amino acid (9) TYPE: amino acid (10) TOPOLOGY: linear (11) TYPE: amino acid (12) TYPE: amino acid (13) TYPE: amino acid (14) TYPE: amino acid (15) TYPE: amino acid (16) TYPE: amino acid (17) TYPE: amino acid (18) TYPE: amino acid (19) TYPE: amino acid (10) TYPE: amino acid (11) TYPE: amino acid (12) TYPE: amino acid (13) TYPE: amino acid (14) TYPE: amino acid (15) TYPE: amino acid (16) TYPE: amino acid (17) TYPE: amino acid (18) TYPE: amino acid (19) TYPE: amino acid (19) TYPE: amino acid (10) TYPE: amin	(A) LENGTH: 26 DASCE PAITS (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:	52
yel Pro Phe Asn Glu Val (2) INFORMATION FOR SEQ ID NO:2: (2) INFORMATION FOR SEQ ID NO:2: (3) INFORMATION FOR SEQ ID NO:2: (4) SEQUENCE CHARACTERISTICS: (5) TYPE: amino acids (6) STRANDEDNESS: Not Applicable (7) STRANDEDNESS: Not Applicable (8) TYPE: amino acids (9) TOPOLOGY: linear (10) TOPOLOGY: linear (11) SEQUENCE DESCRIPTION: SEQ ID NO:2: (12) TYPE: amino acids (13) TYPE: amino acids (14) TYPE: amino acids (15) TYPE: amino acids (16) TYPE: amino acids (17) TYPE: amino acids (18) TYPE: amino acids (19) TYPE: amino acids (19) TYPE: amino acids (10) TYPE: amino acids (1	(2) INFORMATION FOR SEQ ID NO:3:	SO
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s 2 In the the GLY GLU GLE Lys Ala His GLU Val	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 amino acids (B) TYPE: amino acid	01
CLON OF OTE INOTESTAGES SAMESTED		ş

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		09
(i) SEQUENCE CHARACTERISTICS: (b) TYPE: nucleic acid (c) STRANDEDNESS: single (d) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11: (xi) SEQUENCE DESCRIPTION: CEQ ID NO:11:	_	57
TTTGTCGACA TACTCCTGGA AGATGTCC 28 INFORMATION FOR SEQ ID NO:11:	(2)	01
(i) ZEŌNENCE DESCRIBLICN: ZEŌ ID NO:10: (B) TYPE: nucleic acid (C) STRANDEDNESS: single (C) STRA	(7)	32
(D) TOPOLOGY: Linear (Xi) SEQUENCE DESCRIPTION: SEQ ID NO:9: TTTGTCGACA ACACAGGACG GCTTGAAG 28 (D) TOPOLOGY: Linear	(2)	OE
INFORMATION FOR SEQ ID NO:9: (1) SEQUENCE CHARACTERISTICS: (B) TYPE: nucleic acid (C) STRANDEDNESS: single (C) STRANDEDNESS: single	(Z)	SS
(A) TYPE: nucleic acid (C) STRANDEDNESS: single (Xi) SEQUENCE DESCRIPTION: SEQ ID NO:8: (Xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:		50
INFORMATION FOR SEQ ID NO:8: (1) SEQUENCE CHARACTERISTICS: (A) LENGTH: I7 base pairs	(2)	SI
(i) SEQUENCE CHARACTERISTICS: (k) LENGTH: 25 base pairs (c) STRANDEDNESS: single (c) STRANDEDNESS: single (xi) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7: TTGTCGACG AAAATCACTG TGAGC 25 TTTGTCGACG AAAATCACTG TGAGC 25		0
(N) TOPOLOGY: linear TTTGTCGACA ACACAGGACG GCTTGAAG 28 TTTGTCGACA ACACAGGACG GCTTGAAG 28 TTTGTCGACA ACACAGGACG GCTTGAAG 28	(2)	٤

(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single		ć
TTTGTCGACA CACCCTAATG AAGTGTC 27 INFORMATION FOR SEQ ID NO:17:	(2)	S
(C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SFO ID NO.16.		
(A) LENGTH: 27 base pairs (B) TYPE: nucleic acid		0#
INFORMATION FOR SEQ ID NO:16: (i) SEQUENCE CHARACTERISTICS:	(2)	
(C) STRANDEDNESS: SINGLE (D) TOPOLOGY: Linear (Xi) SEQUENCE DESCRIPTION: SEQ ID NO:15: TTTGTCGACT CRTCRTRCA RCANCC 26		32
(A) LENGTH: 26 base pairs (B) TYPE: nucleic acid		
INFORMATION FOR SEQ ID NO:15: (i) SEQUENCE CHARACTERISTICS:	(2)	0€
(i) SEQUENCE CHARACTERISTICS: (k) LENGTH: 26 base pairs (g) TYPE: nucleic acid (c) STRANDEDNESS: single (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14: (xi) SEQUENCE ATATHGCNG AYGARC 26 TTTGTCGACA TAYATHGCNG AYGARC 26		52
INFORMATION FOR SEQ ID NO:14:	(2)	50
(C) STRANDEDNESS: Single (D) TOPOLOGY: linear (Xi) SEQUENCE DESCRIPTION: SEQ ID NO:13: TTTGTCGACG GTGAGGGTC TAGTTC 26		
(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid		ĝi.
INFORMATION FOR SEQ ID NO:13:	(2)	O!
(C) STRANDEDNESS: SINGLE (XI) SEQUENCE DESCRIPTION: SEQUENC: SEQUENC: (XI) TOPOLOGY: Linear (XI) SEQUENCENCE SEQUENCES SEQUENC		UI.
(1) SEQUENCE CHARACTERISTICS; (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid		S
INFORMATION FOR SEQ ID NO:12:	(2)	

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82 STYTASTS MAASASASTS ASASSTETTT		
(xi) ZEŌNENCE DESCHIBLION: ZEŌ ID NO:55:		
(D) LOBOPOCK: Jinear		
(C) SIFANDEDNESS: single		S\$
(B) LAbE: uncjerc scrq (Y) PENGLH: SR Dyse byrrs		
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs		
INFORMATION FOR SEQ ID NO:22:	(2)	
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TTTGTCGACA TTCAGTCCNT CNTGYGT 27		
(XI) SEĞNENCE DESCEIBLION: SEĞ ID NO:SI:		
(D) LOBOFOCK: Jinear		
(C) STRANDEDNESS: single		
(B) LLbE: uncjeje gejq (V) FENCLH: 5\ pgze bgitz		32
(i) SEQUENCE CHARACTERISTICS: (A)		
INFORMATION FOR SEQ ID NO:21:	(2)	
VICTOR SI SIS		
82 SEBASA TAAATAATON NOASSTETTT		30
(XI) ZEĞNENCE DEZCEIBLION: ZEĞ ID NO:50:		
(D) TOPOLOGY: linear		
(C) ZLKYNDEDNEZZ: zrudje (B) LLbe: uncierc scra		
(i) SEQUENCE CHARACTERISTICS: (A)		52
INFORMATION FOR SEQ ID NO:20:	(2)	
00 0K C2 000 ===		
TTTGTCGACN NGCAGGTCCT AGCTG 25		SO
(xt) SEQUENCE DESCRIPTION: SEQ ID NO:19:		
(D) LOBOTOGA: Truest		
(C) SLEYNDEDNESS: single (B) TYPE: nucleic acid		
(A) LENGTH: 25 base pairs		
(!) REQUENCE CHARACTERISTICS:		Si
INFORMATION FOR SEQ ID NO:19:	(2)	
(x1) SEQUENCE DESCRIPTION: SEQ ID NO. 10.		
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(C) ZIEANDEDNESS: single (C) TOPOLOGY: Linear		
(B) TYPE: nucleic acid		
(Y) TENCLH: 51 pase parts		
(I) SEQUENCE CHARACTERISTICS:	4 1	
INFORMATION FOR SEQ ID NO:18:	(2)	9
TITOTOPIC PROPERTY UNDO TO TIT		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO.11.		
(xţ) ZEŌNENCE DESCHIBLION: ZEŌ ID NO:J\: (D) LOBOFOGK: JŢUGGI		

5	(2)	INFORMATION FOR SEQ ID NO:23: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23: TTTGTCGACA CTGCACTGTG TGCCGGTG 28
15	(2)	INFORMATION FOR SEQ ID NO:24: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single
		(D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24: TTTGTCGACA ACATTGGCCG TCTCCACC 28
20	(2)	INFORMATION FOR SEQ ID NO:25: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single
25		(D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25: TTTGTCGACA ATCGCCGCAG CAGCCGGT 28
30	(2)	INFORMATION FOR SEQ ID NO:26: (i) SEQUENCE CHARACTERISTICS: - (A) LENGTH: 27 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear
35		(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26: TTTGTCGACT GGCTCTGGAC GTCTGAG
40	(2)	INFORMATION FOR SEQ ID NO:27: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single
45		(D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27: TTTGTCGACA CTGAAGAGTG TGACGG 26
	(2)	INFORMATION FOR SEQ ID NO:28: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 51 base pairs (B) TYPE: nucleic acid
50		(C) STRANDEDNESS: single

		(xi			SY: lin SCRIPTI		O ID 1	10:28:
5	GCT GI		CCC ACA	GAC TG	C CAC C	TG TGC	GGC G	AT
10	51	CCC CGG	AGG TAA			·		
	(2) I	NFORMAT (i)			RACTERI	STICS:		
15		ix)	(B)	TYPE: STRANDE TOPOLOG	amino a EDNESS: GY: lir	single ear		NO:29:
20	Ala Va		Pro Thr	Asp Cy	s His L	eu Cys	Gly A	Asp
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25		Pro Arg 15 INFORMAT (i)	ION FOR	NCE CHA	RACTERI			
<i>30</i>	AACC	(x :	(B)	TYPE: STRANDI TOPOLO	: 577 b nucleic EDNESS: GY: lir ESCRIPTI	acid single mear	e	NO:30:
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35	Met Asr	n Phe Leu	CTC TCT Leu Ser 5	Trp Val	His Trp	Thr Leu	Ala L	TA CTG 49 eu Leu 15
								CG ACA 94
40	Leu Tyr	r Leu His	His Ala 20	Lys Trp	Ser Gln 25	Ala Ala	Pro T	hr Thr 30
								AC GTC 139
	Glu Gly	y Glu Gln	Lys Ala 35	His Glu	Val Val	Lys Phe	Met A	sp Val 40
45			TAT TGC Tyr Cys 50					AC ATC 184 sp Ile 60
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50			Pro Asp					

	TG Cy	T GT s Va	G CC	C CTA	HIEL	Arg	TGT Cys	GCG Ala	GGC Gly	TGC Cys	TGC	AAT Asn	GAT Asp	GAA Glu	GCC	274
5				C GTG	80					85	1				90	
		• ••	u cy.	s val	95	Thr	Ser	Glu	Ser	100	. Val	Thr	Met	Gln	Ile 105	
10			,	C AAA E Lys	110	ura	GIN	ser	GIN	H15	Ile	Gly	Glu	Met	Ser 120	
15	TT: Phe	CTC	G CAC	CAT His	AGC Ser 125	AGA Arg	TGT Cys	GAA Glu	TGC Cys	AGA Arg 130	CCA Pro	AAG Lys	AAA Lys	GAT Asp	AGA Arg 135	409
	ACA Thr	AAC Lys	G CCA B Pro	GAA Glu	AAT Asn 140	CAC His	TGT Cys	GAG Glu	CCT Pro	TGT Cys 145	TCA Ser	GAG Glu	CGG Arg	AGA Arg	AAG Lys 150	454
20	CAT His	TTC	TTT Phe	GTC Val	CAA Gln 155	GAT Asp	CCG Pro	CAG Gln	ACG Thr	TGT Cys 160	AAA Lys	TGT Cys	TCC Ser	TGC Cys	AAA Lys 165	499
25	AAC Asn	ACA Thr	GAC Asp	TCG Ser	CGT Arg 170	TGC Cys	AAG Lys	GCG Ala	AGG Arg	CAG Gln 175	CTT Leu	GAG Glu	TTA Leu	AAC Asr	GAA Glu 180	544
30	CGT Arg	ACT Thr	TGC Cys	AGA Arg	TGT Cys 185	GAC Asp	AAG Lys	CCA Pro	AGG Arg	CGG Arg 190	TGA					577
	(2)	I	NFOF	(i)	SE (A	QUEI	NCE LENG	CHA TH:	RACT	TERI 90 a	STI		:ids			
35				(xi)	(c) :	TYPE STRA TOPO ENCE	NDE	DNES Y:	SS: lin	si: ear	ngle SEQ		NO:	31:	
40	Met	Asn	Phe	Leu	Leu S	Ser	Trp '	Val 1	His	Trp 10	Thr	Leu .	Ala	Leu	Leu 15	
	Leu-	Tyr	Leu	His :	His A	Ala 1	Lys :	Trp :	Ser	Gln . 25	Ala	Ala 1	Pro '	Thr	Thr 30	
15	Glu	Gly	Glu	Gln :	Lys # 35	Ala i	dis (Glu V	/al v	Val :	Lys :	Phe I	Met i	Asp '	Val 45	
	Tyr	Gln	Arg	Ser :	5 0	ys A	Arg E	Pro 1	le (3lu : 55	Thr 1	Leu 1	/al /	Asp :	Ile 60	
50	Phe	Gln	Glu	Tyr I	Pro A	sp (lu 1	le G	3lu 1	lyr :	le i	Phe 1	Lys I	Pro S	Ser	

					65					70					75	
5	Cys	Val	Pro	Leu	Met 80	Arg	Суз	Ala	Gly	Cys 85	Cys .	Asn	Asp	Glu	Ala 90	
	Leu	Glu	Cys	Val	Pro 95	Thr	Ser	Glu	Ser	Asn 100	Val	Thr	Met	Gln	Ile 105	
10	Met	Arg	Ile	Lys	Pro 110	His	Gln	Ser	Gln	His 115	Ile	Gly	Glu	Met	Ser 120	
	Phe	Leu	Gln	His	Ser 125	Arg	Cys	Glu	Cys	Arg 130	Pro	Lys	Lys	Asp	Arg 135	
15	Thr	Lys	Pro	Glu	Asn 140	His	Cys	Glu	Pro	Cys 145	Ser	Glu	Arg	Arg	Lys 150	
	His	Leu	Phe	Val	Gln 155	Asp	Pro	Gln	Thr	Cys 160	Lys	Суз	Ser	Cys	Lys 165	
20	Asn	Thr	Asp	Ser	Arg 170		Lys	Ala	Arg	Gln 175	Leu	Glu	Leu	Asn	Glu 180	
	Arg	Thr	Cys	Arg	Cys 185		Lys	Pro	Arg	Arg 190						
25	(2) INFORMATION FOR SEQ ID NO:32: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 445 base pairs (B) TYPE: nucleic acid															
30				(xi	Ò	C) D) SEQ	TOF	OLO	GY:		near			on c	:32:	
	ACC.	A														4
35	ATG Met	AAC Asn	TTI Phe	CTG Leu	CTC Leu	Ser	TGG Trp	GTG Val	CAC His	TGG Trp	Thr	CTG	GCT	TTA Leu	CTG Leu 15	49
40	CTG Leu	TAC	CTC Lev	CAC His	CAT His	Ala	AAG Lys	TG	TCC Sea	CAC Glr 25	Ala	GCA Ala	Pro	ACG Thr	ACA Thr 30	94
	GAA Glu	GG(G GAC	G CAG	AAA Lys	a Ala	CAT His	GAJ	A GTO	G GT(1 Val 4(Lys	TTC Phe	ATC Met	GAC Asp	GTC Val 45	139
45	TAC Tyr	CAC Gli	G CGC	C AGO Ser	TA:	Cy:	C CG1	CCC	S AT	T GAG e Gli 5	ı Thr	CTC Lev	GTO Val	G GAC L Asp	ATC Ile 60	184
50	TTC Phe	CAC	G GAG	TAC	CC	C GA	T GAG	AT	A GA	G TA'	r ATC	TTC Phe	Ly:	G CCC	TCC Ser	229

					6.5	5				7()				75	
5	TGI Cys	GTC Val	CCC.	CTA Leu	ATO Met	Arç	TGT Cys	GCG	GG(TGC Cys	Cys	AAT Asn	GA1	GAF Glu	GCC Ala 90	274
10	CTG Leu	GAG Glu	TGC Cys	GTG Val	Pro 95	THE	TCG Ser	GA0	AGC Ser	AAC Asn 100	Val	ACT	ATC Met	Gln	ATC Ile 105	319
	ATG Met	CGG	Ile	AAA Lys	Pro 110	ura	CAA Gln	AGC Ser	CAC Glr	CAC His	Ile	GGA Gly	GAG Glu	ATG Met	AGC Ser 120	364
15	TTC Phe	CTG Leu	CAG Gln	CAT His	AGC Ser 125	Arg	TGT Cys	GAA Glu	TGC	AGA Arg 130	Pro	AAG Lys	AAA Lys	GAT Asp	AGA Arg 135	409
20	ACA Thr	AAG Lys	CCA Pro	GAA Glu	AAA Lys 140	Cys	GAC Asp	AAG Lys	CCA Pro	AGG Arg 145	CGG Arg	TGA				445
25	(2)	I	NFOF	TAMS (i)	S:	FOR EQUE A) B)	LEN	CH! GTH	ARAC : 1	TER	ISTI amin		cid	S		
				(xi	(C) D)	STR. TOP	AND!	EDNE SY:	SS:	si near	nglo SE() NO	:33:	
30	Met	Asn	Phe	Leu	Leu 5	Ser	Trp	Val	His	Trp 10	Thr	Leu	Ala	Leu	Leu 15	
35					20					25	Ala				30	
35					33					40	Lys				45	
40					50		•			55	Thr				60	
	Phe				65					70					75	
45	Суз				80					85					90	
	Leu				95					100					105	
50	Met 1	Arg	Ile	Lys	Pro 110	His	Gln :	Ser		His 115	Ile	Gly	Glu		Ser 120	

	Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg 135														
5	Thr Lys Pro Glu Lys Cys Asp Lys Pro Arg Arg 140 145														
10	(2) INFORMATION FOR SEQ ID NO:34: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 649 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:														
75	AACC														
20	ATG AAC TTT CTG CTC TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG 49 Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu 15														
	CTG TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ACG ACA 94 Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr 20 25 30														
25	GAA GGG GAG CAG AAA GCC CAT GAA GTG GTG AAG TTC ATG GAC GTC 139 Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val 35 40														
30	TAC CAG CGC AGC TAT TGC CGT CCG ATT GAG ACC CTG GTG GAC ATC 184 Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile 50 60														
35	TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TCC 229 Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser 70 75														
3.5	TGT GTG CCC CTA ATG CGG TGT GCG GGC TGC TGC AAT GAT GAA GCC 274 Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala 80 85														
40	CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC GTC ACT ATG CAG ATC 319 Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile 95 100 105														
45	ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC 364 Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser 110 115														
	TTC CTG CAG CAT AGC AGA TGT GAA TGC AGA CCA AAG AAA GAT AGA 409 Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg 135														
50	ACA AAG CCA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGT CAA 454														

	Thr Lys Pro Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln 140 . 145 . 150														
5	AAA CGA AAG CGC AAG AAA TCC CGG TTT AAA TCC TGG AGC GTT CAC 499 Lys Arg Lys Arg Lys Ser Arg Phe Lys Ser Trp Ser Val His 155 160 165														
10	TGT GAG CCT TGT TCA GAG CGG AGA AAG CAT TTG TTT GTC CAA GAT 544 Cys Glu Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp 170 175 180														
	CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC 569 Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys 185 190 195														
15	AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC 634 Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp 200 205 210														
20	AAG CCA AGG CGG TGA Lys Pro Arg Arg 649														
25	(2) INFORMATION FOR SEQ ID NO:35: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 214 amino acids (B) TYPE: amino acid														
20															
30	Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu 5 10 15														
35	Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr 20 25 30 Glu Gly Glu Glo Lys Ala Ric Clu Talana														
	Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val 35 40 45 Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile														
40	Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser														
45	Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala 80 85 90														
	Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile 95 100 105														
50	Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser 110 115 120														

	Phe	Leu	Gln	His S	Ser A	rg (Cys	Glu	Суз	Arg 130	Pro 1	ys :	Lys	Asp	Arg 135	
5	Thr	Lys	Pro	Glu !	Lys I 140	Lys :	Ser	Val	Arg	Gly 145	Lys (Sly	Lys	Gly	Gln 150	
	Lys	Arg	Lys	Arg :	Lys 1 155	Lys	Ser	Arg	Phe	Lys 160	Ser	rp	Ser	Val	His 165	
10	Суз	Glu	Pro	Cys	Ser (Glu	Arg	Arg	Lys	His 175	Leu	Phe	Val	Gln	Asp 180	
	Pro	Gln	Thr	Суз	Lys 185	Cys	Ser	Суз	Lys	Asn 190	Thr	Asp	Ser	Arg	Cys 195	
15	Lys	Ala	Arg	Gln	Leu 200	Glu	Leu	Asn	Glu	Arg 205	Thr	Суз	Arg	Cys	Asp 210	
	Lys	Pro	Arg	Arg												
20	(2)	I	NFOI	RMAT:	SE (1 (1	QUE A) B) C)	NCE LEN TYP STR	CHI GTH E: AND	ARAC : 'u : nuc EDNI	TER 417 clei ESS:	ISTI base c ac	pa id ngl				
25				(xi) `\$		JENC		ESCI	RIPT	ION:	SE			36:	
	ATC Met	CTC	G GCC	ATG Met	AAG Lys 5	CTG Leu	TTC Phe	ACT Thr	TGC Cys	TTC Phe	Leu	Gln	GTC Val	CTA L Le	A GCT L Ala 15	45
30	GG(Gl ₃	G TT	G GC1	r GTG a Val	CAC His 20	TCC	CAG Gln	GGG Gly	GC0	CTC a Leu 25	Ser	GCT Ala	GG(AA S Y As	C AAC n Asn 30	90
35	TC: Se:	A AC	A GAI	A ATG	GAA Glu 35	GTG Val	GTC L Val	CC1	TTO Ph	C AAS e Asi 4	n Giv	GTC Val	TG	G GG p Gl	C CGC y Arg 45	135
40	AG Se	C TA r Ty	C TG	C CGG s Arg	CCA Pro	Met	G GAC	AA(G CT	G GT u Va 5	T TAI	AT:	r GC e Al	A GA a As	T GAA p Glu 60	
	CA Hi	C CC	T AA to As	T GAA	GTG Val	Se	T CA' r Hi	T AT.	A TT e Ph	e se	T CCC r Pro	TC. Se	A TG	T GT	C CTT 1 Leu 75	
45	CT Le	G AC	ST CG	C TG	T AGT sesses sesses	G1;	C TG y Cy	C TG s Cy	T GO	y As	C GAG p Gl	G GG	T CI y Le	rG Ci	AC TGT is Cys	•

	G V	TG	GCG Ala	CTA	AA(ACA Thi	A GCC	AA S AS	C A'n I	TC A le T	*** L	ATG (CAG I	ATC [le	TTA Leu	AA(Lys	G ATT	315
5	C P	CC (CCC Pro	AAT Asn	CGG	GAT Asp 110		CA'	T T(CC T	AC G	TG G	iAG A	ATG let	ACA Thr	TTC Phe	105 TCT Ser	360
10	C	AG (GAT	GTA	CTC	TGC Cys	GAA Glu	TC				.15					120 AAG Lys	405
	GC	A G	AA		TAA	125					1	30			****	III	135	417
15	(2			_	MAT (i)	ION SI	FOR EQUE	SE NCE	Q I	D N	O:3	7:	TTC	z .				
20					(xi	() () ()	A) B) C) O)	TYP STR TOP	IGTI E: LANI OLC	H: ami DEDN DGY:	138 .no IESS	am aci :	ino d sino	ac: gle		NO	: 37 :	
25						Lys 5	Leu	Phe	Thi	Cy:	s Ph	e Le	u Gl	ת.	al :	Leu	Ala 15	
30						His 20 Glu '					As	5 n G1					30	
						Pro 1					4	0 1 Ty .					45	
35						7al S 65					/ (,					Leu 75	
						er G 80					53	,					90	
40						hr A 95					100	,				1	.05	
45					_	sp P 10					112					1	20	
	Gln Ala				1.	ys G. 25	ru C	ys A	irg	Pro	Ile 130	Leu	Glu	Th	r Tì		ys 35	
50	(2)			RMA	TIO	n fo Sequ	OR S UENC	EQ CE (ID CHAI	NO:	38: ER:	STI	cs:					

	(A) LENGTH: 477 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:															
5				(xi)	-	EQUI	ENCE	DE	SCRI	PTI	ON:	SEQ	ID	NO:	38:	
	ATG Met	CTG Leu	GCC Ala	ATG . Met	AAG (Lys 5	CTG Leu	TTC Phe	ACT Thr	TGC Cys	TTC Phe 10	TTG Leu	CAG Gln	GTC Val	CTA Leu	GCT Ala 15	45
10	GGG Gly	TTG Leu	GCT Ala	GTG Val	CAC His 20	TCC Ser	CAG Gln	G17 GGG	GCC Ala	CTG Leu 25	TCT Ser	GCT Ala	GGG Gly	AAC Asn	AAC Asn 30	90
15	TCA Ser	ACA Thr	GAA Glu	ATG Met	GAA Glu 35	GTG Val	GTG Val	CCT Pro	TTC Phe	AAT Asn 40	GAA Glu	GTG Val	TGG Trp	GGC	CGC Arg 45	135
20	AGC Ser	TAC	TGC Cys	CGG Arg	CCA Pro 50	ATG Met	GAG Glu	AAG Lys	CTG Leu	GTG Val 55	TAC Tyr	ATT Ile	GCA Ala	GAT Asp	GAA Glu 60	180
	CAC His	CCT Pro	AAT Asn	GAA Glu	GTG Val 65	TCT Ser	CAT	ATA Ile	TTC Phe	AGT Ser 70	CCG Pro	TCA Ser	TGT Cys	GTC Val	CTT Leu 75	225
25	CTG Leu	AGT Ser	CGC Arg	TGT Cys	AGT Ser 80	GGC Gly	TGC	TGT Cys	GGT Gly	GAC Asp 85	GIU	GGT	CTG Leu	CAC His	TGT Cys 90	270
30	GTG Val	GCG Ala	CTA	AAG Lys	ACA Thr 95	Ala	AAC Asn	ATC	ACT Thr	Met 100	. 611	ATC 11e	: TTA : Leu	AAG Lys	Ile 105	
	CCC	CCC Pro	C AAT AST	CGG	GAT Asp	Pro	CAT His	TCC Ser	TAC Tyr	GT0	LGI	ATC	ACA Thi	TTC Phe	TCT Ser 120	360 :
35	CAG Glr	GA:	r GTI p Val	A CTO	TGC 1 Cys 125	G1	A TGO	C AGG	G CC	T AT:	e ne	G GAG	G ACC	G ACA	AAC Lys	405 3
40	GC# Ala	A GA B G1	A AG	G AGO	3 AAJ g Lys 140	3 Th	C AA	G GGG S Gl	G AA	G AG s Ar 14	ā rā	G CA. s Gl	11 26	C AAI r Ly:		2 450 E 0
45	CC: Pr	A CA o Gl	G AC	T GA r Gl	G GA u Gl	u Pr	C CA o Hi	C CT s Le	G TG	A						477
	(2	!)	INFO		T101 .)	SEC	TENC	EQ I E CI	HARA	CTE	RIS:	rics ino	: aci	ds		
50						•										

5				(x	((B) (C) (D) SEQ	STI	RAND POLO	amii EDNI GY: ESCI	ESS: li	s. nea:	ingl r : SE		D NC):39	:
	Met	t Le	ı Ala	a Met	Lys 5	Leu	Phe	Thr	Cys	Phe 10	Leu	Gln	Val	Leu	Ala 15	
10	Gly	y Lev	Ala	a Val	. His	Ser	Gln	Gly	Ala	Leu 25	Ser	Ala	Gly	Asn	Asn 30	
15	Sei	The	: Glu	Met	Glu 35	Val	Val	Pro	Phe	Asn 40	Glu	Val	Trp	Gly	Arg 45	
15	Sez	Туг	Cys	Arg	Pro 50	Met	Glu	Lys	Leu	Val 55	Tyr	Ile	Ala	Asp	Glu 60	
· 20	His	Pro	Asn	Glu	Val 65	Ser	His	Ile	Phe	Ser 70	Pro	Ser	Суз	Val	Leu 75	
	Leu	Ser	Arg	Суз	Ser 80	Gly	Cys	Cys	Gly	Asp 85	Glu	Gly	Leu	His	Cys 90	
25	Val	Ala	Leu	Lys	Thr 95	Ala	Asn	Ile	Thr	Met 100	Gln	Ile	Leu	Lys	Ile 105	
	Pro	Pro	Asn	Arg	Asp 110	Pro	His	Ser	Tyr	Val 115	Glu	Met	Thr	Phe	Ser 120	
30	Gln	Asp	Val	Leu	Cys 125	Glu	Cys	Arg	Pro	Ile 130	Leu	Glu	Thr	Thr	Lys 135	
	Ala	Glu	Arg	Arg	Lys 140	Thr	Lys	Gly	Lys	Arg 145	Lys	Gln	Ser	Lys	Thr 150	
35	Pro	Gln	Thr	Glu	Glu 155	Pro	His	Leu								
	(2)	Iì	NFOR	MAT: (i)	ION SE	QUE	NCE	CHA	RAC	TERI						
10	-			(xi	(D	3) ' :) '	TOPO	E: ANDE OLOG	40 nuc: DNES Y: SCRI	leic SS: lin	si: ear	id ngle	!	NO:	40:	
5	ATG Met	CCG Pro	GTC Val	ATG Met	AGG Arg 5	CTG	TTC Phe	CCT	TGC :	TTC Phe	CTG (CAG (Gln)	CTC (Leu :	CTG .	GCC Ala 15	45

	GGG Gly	CTG Leu	GCG Ala	CTG Leu	CCT Pro 20	GCT Ala	GTG Val	CCC Pro	CCC Pro	CAG Gln 25	CAG Gln	TGG Trp	GCC Ala	TTG Leu	TCT Ser 30	90
5	GCT Ala	G17 GGG	AAC Asn	GGC Gly	TCG Ser 35	TCA Ser	GAG Glu	GTG Val	GAA Glu	GTG Val 40	GTA Val	CCC Pro	TTC Phe	CAG Gln	GAA Glu 45	135
10	GTG Val	TGG Trp	GGC Gly	CGC Arg	AGC Ser 50	TAC Tyr	TGC Cys	CGG Arg	GCG Ala	CTG Leu 55	GAG Glu	AGG Arg	CTG Leu	GTG Val	GAC Asp 60	180
15	GTC Val	GTG Val	TCC Ser	GAG Glu	TAC Tyr 65	CCC	AGC Ser	GAG Glu	GTG Val	GAG Glu 70	CAC His	ATG Met	TTC Phe	AGC Ser	CCA Pro 75	225
	TCC Ser	TGT Cys	GTC Val	TCC Ser	CTG Leu 80	CTG Leu	CGC	TGC Cys	ACC Thr	GGC Gly 85	Cys	TGC Cys	GGC Gly	GAT Asp	GAG Glu 90	270
20	AAT Asn	CTG Leu	CAC His	TGT Cys	GTG Val 95	Pro	GTG Val	GAG Glu	ACG Thr	GCC Ala 100	Asn	GTC Val	ACC Thr	ATG Met	CAG Gln 105	315
25	CTC Leu	CTA Leu	AAG Lys	ATC Ile	CGT Arg 110	Ser	GGG	GAC Asp	CGG	CCC Pro 115	Ser	TAC	GTG Val	GAG Glu	CTG Leu 120	360
	ACC Thr	TTC Phe	TCI Ser	CAG Gln	CAC His	Val	CGC Arg	TGC Cys	GA.	TGC Cys	Arg	CCT Pro	CTG Lev	CGG Arg	GAG Glu 135	
30	AA(ATO	AAC Lys	CCG Pro	GAP Glu	ı Arç	AGG Arg	G AGA	CCC J Pro	C AAC D Lys 145	5 G13	AGO Arg	G GGG G Gly	AAC Lys	AGG Arg 150	450
35				AAC 1 Lys		3										465
40	(2) :	INFC	RMA') :	SEQU (A) (B) (C) (D)	ENC LE TY ST TO	E CH NGTH PE: RANI POLO	IARA i: ami DEDN DGY:	no i IESS	RIST ami ació : s inea	.no l sing ar	acio le			
45	Me	t Pr	o Va		i) t Ar	SEC	QUEN	CE I	ESC	RIP'					O:41 u Al 1	a
50	Gl	y Le	u Al	a Le	u Pr 2	o Al	a Va	l Pr	o Pi	o G1	.n Gl 25	n Ti	p Al	a Le	u Se 3	r 0

	Ala	Gly	Asn	Gly	Ser 35	Ser	Glu	Val	Glu	Val 40	Val	Pro	Phe	Gln	Glu 45
5	Val	Trp	Gly	Arg	Ser 50	Tyr	Cys	Arg	Ala	Leu 55	Glu	Arg	Leu	Val	Asp 60
	Val	Val	Ser	Glu	Tyr 65	Pro	Ser	Glu	Val	Glu 70	His	Met	Phe	Ser	Pro 75
10	Ser	Суз	Val	Ser	Leu 80	Leu	Arg	Суз	Thr	Gly 85	Cys	Cys	Gly	Asp	Glu 90
	Asn	Leu	His	Cys	Val 95	Pro	Val	Glu	Thr	Ala 100	Asn	Val	Thr	Met	Gln 105
15	Leu	Leu	Lys	Ile	Arg 110	Ser	Gly	Asp	Arg	Pro 115	Ser	Tyr	Val	Glu	Leu 120
20	Thr	Phe	Ser	Gln	His 125	Val	Arg	Суз	Glu	Cys 130	Arg	Pro	Leu	Arg	Glu 135
	Lys	Met	Lys	Pro	Glu 140	Arg	Arg	Arg	Pro	Lys 145	Gly	Arg	Gly	Lys	Arg 150
)E	Arg	Arg	Glu	Lys											

25

Claims

- A purified and isolated DNA sequence encoding the C subunit of vascular endothelial cell growth factor.
 - 2. A purified and isolated vascular endothelial cell growth factor C subunit DNA sequence comprising:
- ATG CCG GTC ATG AGG CTG TTC CCT TGC TTC CTG CAG CTC CTG 35 SCC GGG CTG GCT GCT GTG CCC CAG CAG TGG GCC TIG TOT GOT GGG AAC GGC TOG TOA GAG GIG GAA GIG GIA COO TTC CAG GAA GTG TGG GGC CGC AGC TAC TGC CGG GCG CTG GAG 40 AGG CTG GTG GAC GTC GTG TOC GAG TAC COC AGC GAG GTG GAG CAG ATG TTC AGC CCA TCC TGT GTC TCC CTG CTG CGC TGC ACC GGC TGC TGC GGC GAT GAG AAT CTG CAC TGT GTG CCG GTG GAG ACG GCC AAT GTC ACC ATG CAG CTC CTA AAG ATC CGT TCT GGG 45 GAC CGG CCC TCC TAC GTG GAG CTG ACG TTC TCT CAG CAC GTT CGC TGC GAA TGC CGG CCT CTG CGG GAG AAG ATG AAG CCG GAA AGG AGG AGA COC AAG GGC AGG GGG AAG AGG AGG AGA GAG AAG 50 TAG. SEQ ID NO:40
 - Vascular endothelial cell growth growth factor AC DNA comprising an A subunit DNA sequence and a C subunit DNA sequence.
- Vascular endothelial cell growth growth factor BC DNA comprising a B subunit DNA sequence and a C subunit DNA sequence.

5. A purified and isolated vascular endothelial cell growth factor C subunit DNA sequence comprising:

ATG CCG GTC ATG AGG CTG TTC CCT TGC TTC CTG CAG CTC CTG

COO GOG CTG COT COT GTG COO COO CAG CAG TGG COO 5 TTG TCT GCT GGG AAC GGC TCG TCA GAG GTG GAA GTG GTA CCC TTC CAG GAA GTG TGG GGC CGC AGC TAC TGC CGG GCG CTG GAG AGG CTG GTG GAC GTC GTG TOO GAG TAC OOC AGC GAG GTG GAG 10 CAC ATG TTC AGC CCA TOC TGT GTC TCC CTG CTG CGC TGC ACC GGC TGC TGC GGC GAT GAG AAT CTG CAC TGT GTG CCG GTG GAG ACG GCC AAT GTC ACC ATG CAG CTC CTA AAG ATC CGT TCT GGG GAC CGG CCC TCC TAC GIG GAG CIG ACG TIC TCT CAG CAC GIT 15 CGC TGC GAA TGC CGG CCT CTG CGG GAG AAG ATG AAG CCG GAA AGG AGG AGA COC AAG GGC AGG GGG AAG AGG AGG AGA GAG AAG CAG AGA CCC ACA GAC TGC CAC CTG TGC GGC GAT GCT GTT CCC 20 SEO ID NOS:29 & 40 CGG AGG TAA.

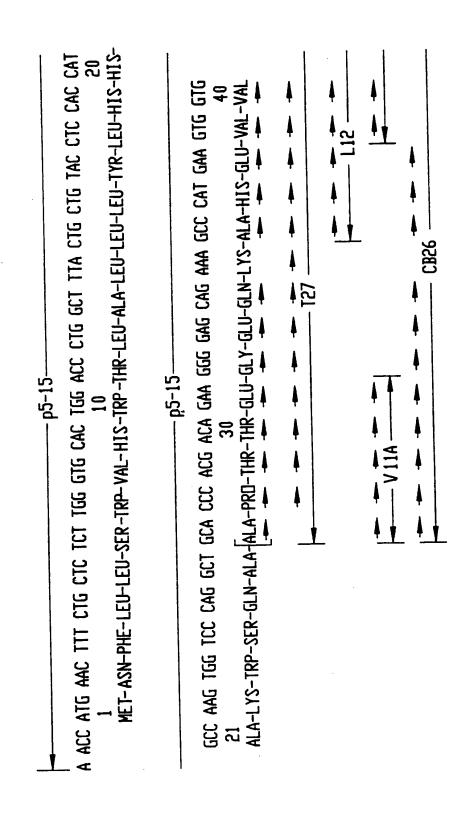
- 6. Vascular endothelial cell growth factor AC DNA comprising an A subunit DNA sequence selected form the group consisting of: a DNA sequence encoding an 189 amino acid form, a DNA sequence encoding an 165 amino acid form and a DNA sequence encoding a 121 amino acid form, with said A subunit DNA operably attached to a C subunit DNA sequence.
- Vascular endothelial cell growth factor BC DNA comprising a B subunit DNA sequence selected form the group consisting of a DNA sequence encoding a 135 amino acid form; and a DNA sequence encoding a 115 amino acid form, with said B subunit DNA sequence operably attached to a C subunit DNA sequence.
 - 8. Homodimeric vascular endothelial growth factor DNA comprising C subunit DNA sequences.
- A vector containing the DNA sequence of any one of claims 3 to 8.
 - 10. A host cell transformed by the vector of claim 9 containing the DNA sequence encoding vascular endothelial cell growth factor.
- A process for the preparation of vascular endothelial cell growth factor comprising culturing the transformed host cell of claim 10 under conditions suitable for the expression of vascular endothelial cell growth factor and recovering vascular endothelial cell growth factor.
 - 12. Vascular endothelial growth factor made by the process of claim 11.
- 45 13. Vascular endothelial cell growth factor AC comprising an A subunit amino acid sequence and a C subunit amino acid sequence.
 - 14. Vascular endothelial cell growth factor BC comprising a B subunit amino acid sequence and a C subunit amino acid sequence.
- 50 15. Vascular endothelial cell growth factor CC comprising a C subunit amino acid sequence and a C subunit amino acid sequence.
 - 16. A purified and isolated vascular endothelial cell growth factor C subunit amino acid sequence comprising:

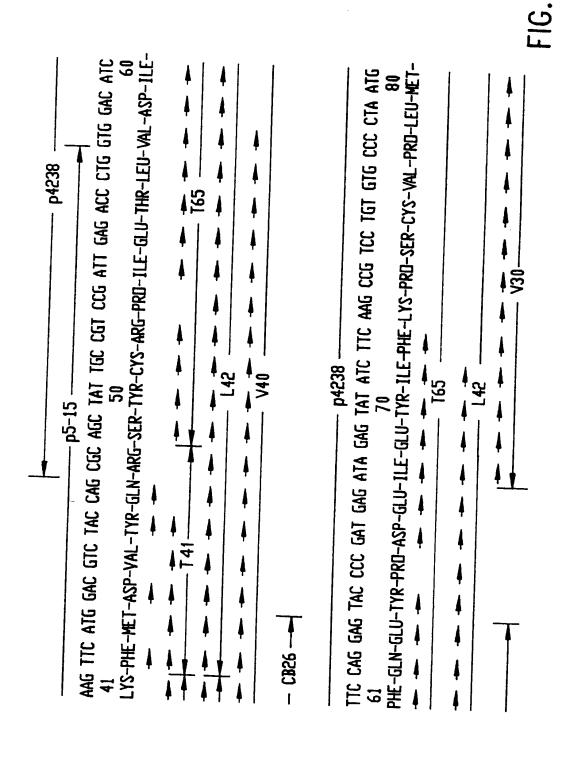
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg Arg Arg Glu Lys. SEQ ID NO:41

20 17. A purified and isolated vascular endothelial cell growth factor C subunit amino acid sequence comprising:

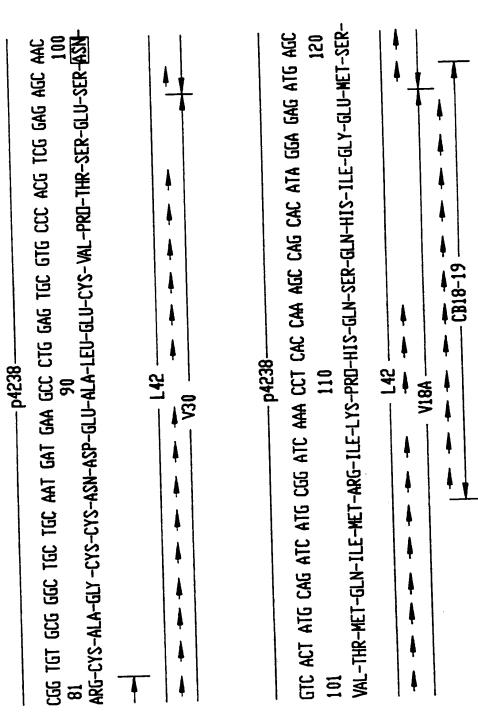
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu
Ala Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala
Leu Ser Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro
Phe Gln Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu
Arg Leu Val Asp Val Val Ser Glu Tyr Pro Ser Glu Val Glu
His Met Phe Ser Pro Ser Cys Val Ser Leu Leu Arg Cys Thr
Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro Val Glu
Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly
Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val
Arg Cys Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu
Arg Arg Arg Pro Thr Asp Cys His Leu Cys Gly Asp Ala Val Pro
Arg Arg Arg. SEQ ID NOS: 29 & 40

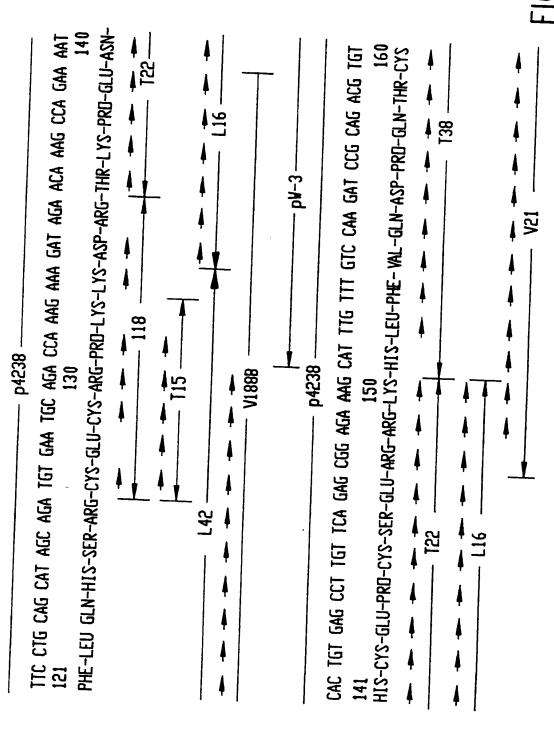
- 18. A tissue repairing pharmaceutical composition comprising a pharmaceutical carrier and an effective tissue repairing amount of the purified vascular endothelial growth factor of any one of claims 13 to 15,
- 19. The use of the vascular endothelial cell growth factor of any one of claims 13 to 15 for the manufacture of a medicament for promoting tissue repair.
- 20. The use of the vascular endothelial cell growth factor of any one of claims 13 to 15 for the manufacture of a medicament for stimulating vascular endothelial cell growth.

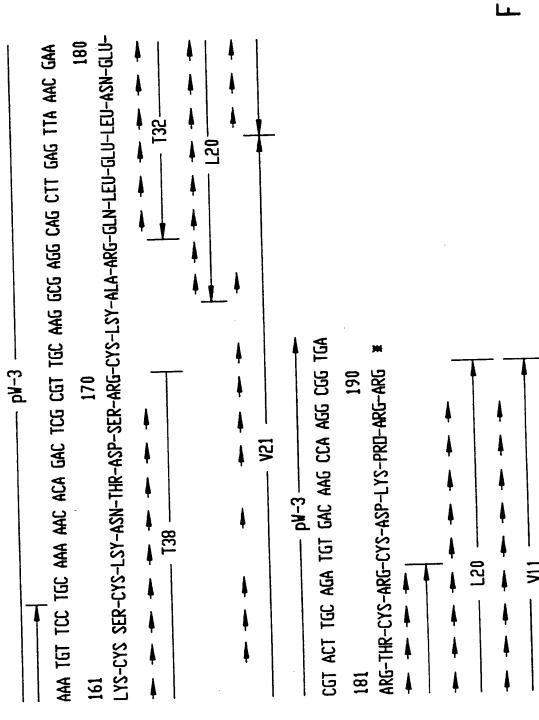


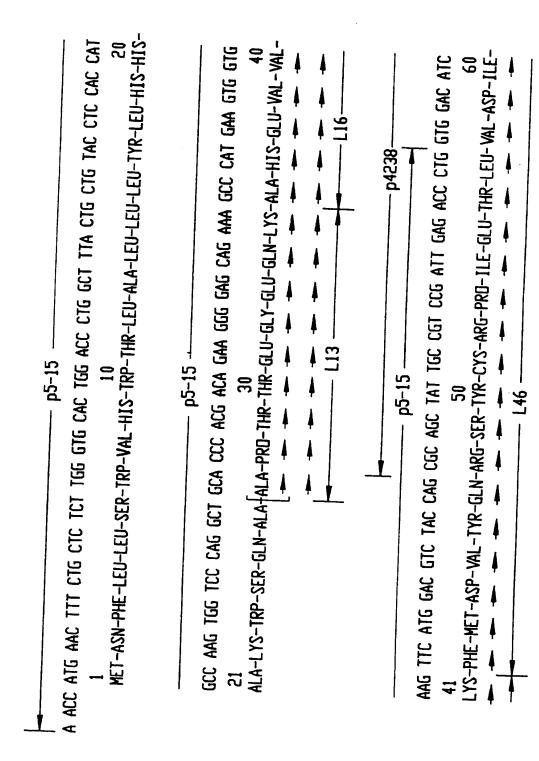




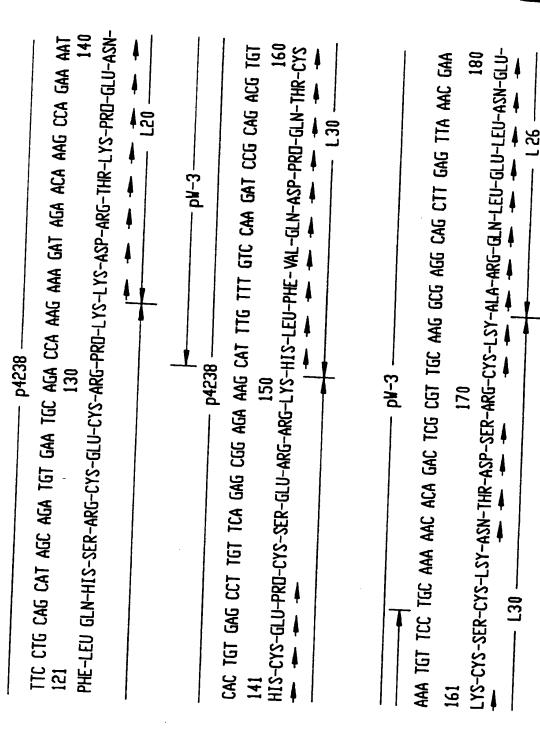


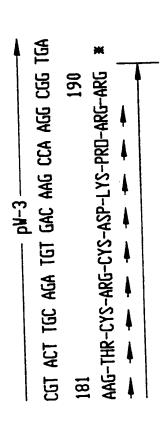


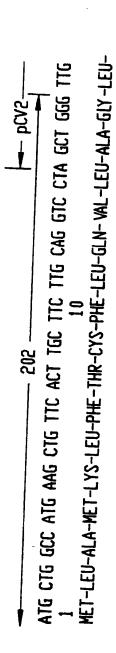


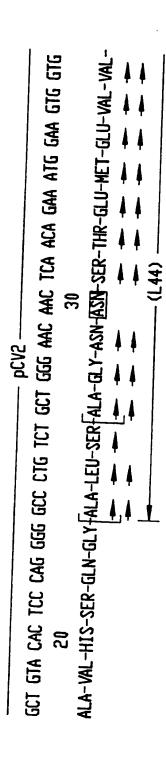


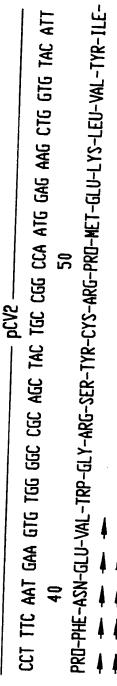
TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TCC TGT GTG CCC CTA ATG 80 A70 PHE-GLN-GLU-TYR-PRO-ASP-GLU-TYR-ILE-PHE-LYS-PRO-SER-CYS-VAL-PRO-LEU-MET-LA6	CGG TGT GCG GGC TGC TGC AAT GAT GAA GCC CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC 100 90 ARG-CYS-ALA-GLV-CYS-CYS-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-LEU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-LEU-GLU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-LEU-GLU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-LEU-GLU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-ALA-CHU-GLU-CYS-VAL-PRD-THR-SER-GLU-SER-ASN-ASP-GLU-CYS-CYS-CYS-CYS-CYS-CYS-CYS-CYS-CYS-CYS	GTC ACT ATG CAG ATC ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC 101 VAL-THR-MET-GLN-ILE-MET-ARG-ILE-LYS-PRD-HIS-GLN-SER-GLN-HIS-ILE-GLY-GLU-MET-SER-
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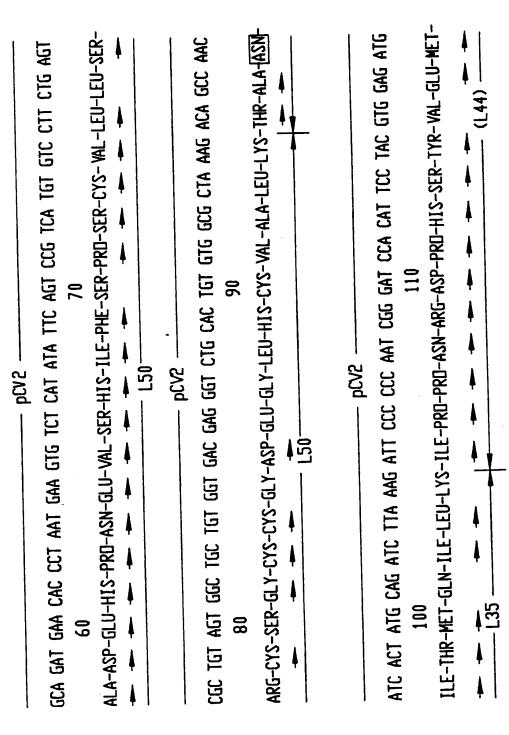


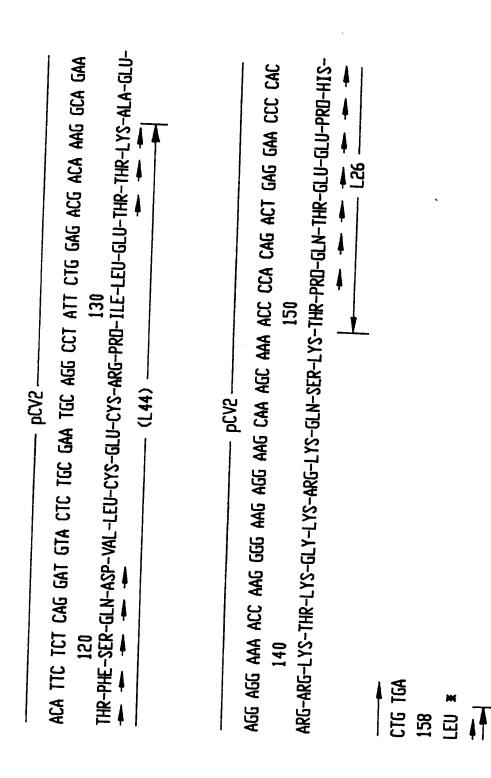












ACCA	ATG Met	AAC Asn	TTT Phe	CIG Leu	CIC Leu 5	TCT Ser	TGG Trp	GIG Val	CAC His	TGG Trp 10	ACC Thr	CIG Leu	CCT Ala	TTA Leu	CTG Leu 15	49
	CTG Leu	TAC Tyr	CTC Leu	CAC His	CAT His 20	GCC Ala	AAG Lys	TGG Trp	TCC Ser	CAG Gln 25	CT Ala	GCA Ala	œ Pro	ACG Thr	ACA Thr 30	94
	GAA Glu	GGG Gly	GAG Glu	CAG Gln	AAA Lys 35	GCC Ala	CAT His	GAA Glu	GIG Val	GIG Val 40	AAG Lys	TTC Phe	ATG Met	GAC Asp	GTC Val 45	139
	TAC Tyr	CAG Gln	CGC Arg	AGC Ser	TAT Tyr 50	TGC Cys	CGT Arg	CCG Pro	ATT Ile	GAG Glu 55	Thr	CIG Leu	GTG Val	GAC Asp	ATC Ile 60	184
	TTC Phe	CAG Gln	GAG Glu	TAC Tyr	CCC Pro 65	GAT Asp	GAG Glu	ATA Ile	GAG Glu	TAT Tyr 70	Ile	TTC Phe	AAG Lys	Pro	TCC Ser 75	229
	TGT Cys	GIG Val	Pro	CTA Leu	ATG Met 80	CGG Arg	TGT Cys	CCG Ala	Gly	TGC Cys 85	Cys	AAT Asn	GAT Asp	GAA Glu	. GCC Ala 90	274
	CTG Leu	GAG Glu	TGC Cys	GIG Val	Pro 95	ACG Thr	TO: Ser	GAG Glu	AGC Ser	AAC AST 100	ı Val	ACT Thr	ATG Met	CAC Glr	ATC lle 105	
	ATC Met	Arg	ATC J Ile	AAA Lys	2 Pro	CAC His	CAA Glr	A AGO n Ser	CAG Glr	CAC His	s Ile	Gly GGZ	A GAC	ATO Met	AGC Ser 120	•
	TTO	CIO Le	G CA(G CAT n His	AGC Ser 125	Arc	IG. Cy:	r GA/ s Glu	A TGC 1 Cys	AG Arg 13	g Pro	A AAC D Lys	G AAI S Lys	A GA' s Asj	T AGA P Arg 135	Ţ
				A GAZ o Gli		s Cys					g An		A			445

AACC	ATG	AAC	. प्रचान	. کلک	- (M)	. 110~1	רייתיים	~ ~	_ ~								
					5	Jei	. 111	, va	1 H.	ls T	10 10	hr	Leu	Ala	Leu	1 Leu 15	1
]	CTG Leu	TAC Tyr	CIC	CAC His	CAT His 20	Ala	AAC Lys	G TG Trj	G TC P Se	EG	AG (ln A 25	CT la	GCA Ala	OCC Pro	ACC Thr	ACA Thr	94
(GAA Glu (GG Gly	GAG Glu	CAG Gln	AAA Lys 35	∞ Ala	CAI His	GA Glu	A GI 1 Va	T V	IG A al L 40	AG ys 1	TTC Phe	ATG Met	GAC Asp	GTC Val 45	139
I	TAC (Tyr (CAG Gln	CCC Arg	AGC Ser	TAT Tyr 50	TGC Cys	CGT Arg	Pro	AT Il	e GI	NG A .u Ti 55	œ (hr I	CIG Leu	GTG Val	GAC Asp	ATC Ile 60	184
T P	MC (Phe G	CAG Sln	GAG Glu	TAC Tyr	CCC Pro 65	GAT Asp	GAG Glu	ATA	GA Glu	עו נ	AT A' T II	IC I	MC he	AAG Lys	CCG Pro	TCC Ser 75	229
Tr C <u>j</u>	GT G ys V	TG al	ecc Pro	CTA Leu	ATG Met . 80	CCG Arg	TGT Cys	CCG Ala	GC	TG Cy:	s C	SC A	AT (sn)	GAT Asp	GAA Glu	GCC Ala 90	274
C: Le	TG G eu G	AG ' lu (IGC Cys I	GTG Val 1	cc . Pro : 95	ACG Thr .	TCG Ser	GAG Glu	AGC Ser	AA Ası 100	n Va	C A 1 T	CT I	ATG Æt (Gln		319
A] Me	IG CO	GG 1 rg 1	ATC I	-,, -, -,	CCT (Pro F	CAC (CAA Gln	AGC Ser	CAG Gln	CAX His	S II	A G e G	GA (AG . Slu !	Met :	AGC Ser .20	364
TI Ph	NC CI	IG (AG (AGC A Ser A 25	AGA :	igi Ys (GAA Glu	TGC Cys	AGZ Arg 130	Pro	A A	AG A vs L	AA (ys <i>l</i>	Asp 1	AGA Arg 35	409
AC Thi	A AA r Ly	NG C rs P	CA G	-u n	AT C sn H 40	AC 1	GT ()ys (GAG Glu 1	CT Pro	TGI Cys 145	Ser	A G2 : G1	AG C	GG A	urg I	NAG 4 Jys 50	154
CA: His	TT SLe	G T u Pl	TT G ne V	TC C al G	AA G ln A	AT C	ro G	AG :	ACG Thr	TGT Cys 160	AA/ Lys	TG Cy	T To	ΩI er C	ys L	AA 4 ys 55	199
AAC Asn	C AC	A G	AC TO SP Se	OG O Pr Au 17	GI IX rg C <u>)</u> 0	3C A /s Ly	AG G ys A	XG / la /	rg GG	CAG Gln 175	CM Leu	GA Gl	G T. u Le	ra a eu A	AC G sn G	lu	44
OGT Arg	CACI	To Cy	C AC S Ar	A TO Cy 185	ST GP VS AS 5	yC Ay To Ly	AG Co ys P:	CA A	rg 1	03G Arg 190	TGA		FI	G	_		77

AACC	ATG Met	AAC Asn	TTT Phe	CIG Leu	CTC Leu 5	TCT Ser	TGG Trp	GIG Val	CAC His	TGG Trp 10	ACC Thr	CIG Leu	GCT Ala	TTA Leu	CTG Leu 15	49
	CIG Leu	TAC Tyr	CTC Leu	CAC His	CAT His 20	GCC Ala	aag Lys	TGG Trp	TCC Ser	CAG Gln 25	CCT Ala	GCA Ala	∞ Pro	ACG Thr	ACA Thr 30	94
	GAA Glu	GG Gly	GAG Glu	CAG Gln	AAA Lys 35	CCC Ala	CAT His	GAA Glu	GTG Val	GIG Val 40	AAG Lys	TIC Phe	ATG Met	GAC Asp	GTC Val 45	139
	TAC Tyr	CAG Gln	CGC Arg	AGC Ser	TAT Tyr 50	TGC Cys	CGT Arg	CCG Pro	ATT Ile	GAG Glu 55	Thr	CTG Leu	GIG Val	GAC Asp	ATC Ile 60	184
	TTC Phe	CAG Gln	GAG Glu	TAC Tyr	occ Pro 65	GAT Asp	GAG Glu	ATA Ile	GAG Glu	TAT Tyr 70	Ile	TTC	: AAG Lys	∝G Pro	TCC Ser 75	229
	TGT Cys	GIG Val	Pro	CTA Leu	ATG Met 80	OGG Arg	TGI Cys	Ala	GCC Gly	TGC Cys 85	Cys	AAI Asn	GAT Asp	GAA Glu	. 600 Ala 90	274
	CTG Leu	GAC Glu	TGC Cys	GIG Val	Pro 95	ACG Thr	TO: Ser	GAG Glu	AGC Ser	AAC Asr 100	Val	ACI Thr	ATG Met	CAG Gln	Ile 105	319
	ATC Met	Arg	ATO	C AA/ e Lys	A CCI Pro 110	CAC His	CA Glr	A AGO	CAC Glr	CAC His 115	s Ile	A GGP e Gly	A GAC	ATC Met	AGC Ser 120	364
	TIC	CIO Leu	G CAM	G CAT n His	r AGC s Ser 125	AGA Arg	i TG I Cys	r GA/ s Glu	A TGC	C AG Arg 13	g Pro	A AA() Lys	G AA/ s Lys	A GAT	AGA Arg 135	409 !
	ACI Thr	A AA Lys	s œ	A GAN	A AAZ 1 Lys 140	Lys	A TC s Se:	A GI r Val	r Œ	A GG g Gl; 14	y Ly:	G GG s Gl	A AA y Ly:	G GG G Gly	r CAV 7 Glr 150	1
	AA) Lys	A CG S Arc	A AA g Ly	G OG s Ar	C AA(g Lys 155	Ly	A TC s Se	C CG r Ar	g TT g Ph	T AA e Ly 16	s Se	C TG	G AG p Se:	C GI r Va	T CAG 1 His 165	5

FIG. 6A

TGT GAG Cys Gli	G CCI 1 Pro	TGI Cys	TCA Ser 170	GAG Glu	CGG Arg	AGA Arg	AAG Lys	CAT His 175	Leu	TTT Phe	GTC Val	CAA Gln	GAT Asp 180	544
OCG CAG Pro Glr	ACG Thr	TGT Cys	AAA Lys 185	TGT Cys	TCC Ser	TGC Cys	AAA Lys	AAC Asn 190	ACA Thr	GAC Asp	TCG Ser	OGT Arg	TGC Cys 195	589
AAG GOG Lys Ala	AGG Arg		CTT Leu 200	GAG Glu	TTA Leu	AAC Asn	GAA Glu	OGT Arg 205	ACT Thr	TGC Cys	AGA Arg	Cys	GAC Asp 210	634
AAG CCA Lys Pro	AGG Arg	CGG Arg	TGA											649

FIG.6B

ATG Met	CTG Leu	GCC	: ATC Met	L	AG (ys I 5	CTG Leu	TTC Phe	ACT Thr	TGC Cys	TTC Phe 10	TIG Leu	CAG Gln	GTC Val	CTA Leu		45
GGG Gly	TIG Leu	GCT Ala	GIV a Va	1 H	AC :	ICC Ser	CAG Gln	Gly	GCC Ala	CTG Leu 25	TCT Ser	GCT Ala	GGG	AAC Asn	AAC Asn 30	90
TCA Ser	ACA Thr	GAN	A AT 1 Me	t G	AA lu 35	GTG Val	GTG Val	CCT Pro	TTC Phe	AAT Asn 40	GAA Glu	GIG Val	TGG Trp	Gly	CCC Arg 45	135
AGC Ser	TAC Tyr	TG Cy	C CG s Ar	g P	CA Pro 50	ATG Met	GAG Glu	AAC Lys	CIG Leu	GTG Val 55	lyr	ATI Ile	GCA Ala	GAT Asp	GAA Glu 60	180
CAC His	CI Pro	AA 1 AS	T G# n Gl	lu V	FIG Val 65	TCT Ser	CAT His	ATA Ile	TTC Phe	AGI Ser 70	Pro	S TCF	TGI Cys	GIC Val	CTT Leu 75	225
CIO	G AG 1 Se	I CO	ic in	ys :	AGT Ser 80	GGC	TGC Cys	TG: Cy:	r cci	CAC TEA Y	GII	G GG G Gly	CIO	G CAC L His	TGT S Cys 90	270
GIV Va	G GC 1 Al	G CI a Le	TA A eu L	AG . ys	ACA Thr 95	GCC	: AAC 1 Asi	C AT	C AC e Th	r ATO	C GI	G AT	C TI	A AA u Ly	G ATT s Ile 105	315
∝ Pr	c cc o Pr	C A	AT C sn A	rg	GAT Asp 110	CZ Pro	A CA'	T TC s Se	C TA	C GT r Va 11	1 GI	G AT u Me	G AC	A TI r Ph	C TCT e Ser 120	360
CA G1	.G G2 .n As	AT G Sp V	TA C al I	eu	100 Cys 125	GA Gl	A TG u Cy	C AC	g Pr	T AT to Il	e re	ic Gr eu Gl	G AC TI U	G AC	A AAG nr Lys 135	405
	A G			'AA												417

FIG.7

ATG CTG GCC ATG AAG CTG TTC ACT TGC TTC TTG CAG GTC CTA GC Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala 5	a S
GGG TTG GCT GTG CAC TCC CAG GGG GCC CTG TCT GCT GGG AAC AAC Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asr 20 25 30	ו
TCA ACA GAA ATG GAA GTG GTG CCT TTC AAT GAA GTG TGG GGC CGC Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg 35 40	Ī
AGC TAC TGC CGG CCA ATG GAG AAG CTG GTG TAC ATT GCA GAT GAA Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu 50 55 60	
CAC CCT AAT GAA GTG TCT CAT ATA TTC AGT CCG TCA TGT GTC CTT His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu 65 70 75	225
CTG AGT CGC TGT AGT GGC TGC TGT GGT GAC GAG GGT CTG CAC TGT Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys 80 85 90	270
GTG GCG CTA AAG ACA GCC AAC ATC ACT ATG CAG ATC TTA AAG ATT Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile 95 100 105	315
CCC CCC AAT CGG GAT CCA CAT TCC TAC GTG GAG ATG ACA TTC TCT Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser 110 120	360
CAG GAT GTA CTC TGC GAA TGC AGG CCT ATT CTG GAG ACG ACA AAG Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys 125 130 135	405
GCA GAA AGG AGG AAA ACC AAG GGG AAG AGG AAG CAA AGC AAA ACC Ala Glu Arg Arg Lys Thr Lys Gly Lys Arg Lys Gln Ser Lys Thr	450
CCA CAG ACT GAG GAA CCC CAC CTG TGA Pro Gln Thr Glu Glu Pro His Leu 155	477

FIG. 8

ATG CCG GTC ATG AGG CTG TTC CCT TGC TTC CTG CAG CTC CTG GCC Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala 5	45
GGG CTG GCG CCT GCT GTG CCC CAG CAG TGG GCC TTG TCT Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser 20 25 30	90
GCT GGG AAC GGC TCG TCA GAG GTG GAA GTG GTA CCC TTC CAG GAA Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu As 35	135
GTG TGG GGC CGC AGC TAC TGC CGG GCG CTG GAG AGG CTG GTG GAC Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp 50 55 60	180
GTC GTG TCC GAG TAC CCC AGC GAG GTG GAG CAC ATG TTC AGC CCA Val Val Ser Glu Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro 65	225
TCC TGT GTC TCC CTG CTG CGC TGC ACC GGC TGC TGC GGC GAT GAG Ser Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu 80 85	270
AAT CTG CAC TGT GTG CCG GTG GAG ACG GCC AAT GTC ACC ATG CAG Asn Leu His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Gln 95 100	315
CTC CTA AAG ATC CGT TCT GGG GAC CGG CCC TCC TAC GTG GAG CTG Leu Leu Lys Ile Arg Ser Gly Asp Arg Pro Ser Tyr Val Glu Leu 110 115 120	360
ACG TTC TCT CAG CAC GTT CGC TGC GAA TGC CGG CCT CTG CGG GAG Thr Phe Ser Gln His Val Arg Cys Glu Cys Arg Pro Leu Arg Glu 125	405
AAG ATG AAG COG GAA AGG AGG AGA COC AAG GGC AGG GGG AAG AGG Lys Met Lys Pro Glu Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg 140 145	,
AGG AGA GAG AAG TAG Arg Arg Glu Lys	465

FIG. 9



EUROPEAN SEARCH REPORT

Application Number

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Place	of south	Date of completion of the search		
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CATE	CORY OF CITED DOCUMENTS			-
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	or the same category cal background	L : document cited in	the application	1

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